

A protease cascade regulates egress
of *Plasmodium falciparum* from the
human erythrocyte

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This thesis is presented for the degree of
Doctor of Philosophy

University College London

12th March 2018

I, James A Thomas confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Impact Statement

During the clinically relevant stage of the malaria parasite's lifecycle in a human host, the asexual blood stage cycle, the parasite invades and replicates within red blood cells before the red blood cell ruptures to release the progeny parasites. This process of parasite release, called egress, is actively controlled by the parasite. Egress has been known to be protease dependent for more than 25 years but the precise proteases mediating the cellular events during egress have remained unknown. Herein is presented the work from my PhD project which ascribes some of the physico-mechanical events during egress to two essential proteases of the malaria parasite. This work is the first to ascribe any of the events of egress to specific effectors during the asexual blood stage cycle. Proteases are attractive targets for the rational design of drugs due to their well characterised mechanisms and active site structures. It is possible that the two proteases investigated in this project can be targeted with small molecule inhibitors with the aim of developing novel antimalarial drugs. There is a great need for novel antimalarial drugs due to extensive resistance to previously used antimalarial compounds and the emergence of resistance to our current frontline artemisinin based chemotherapies.

Additionally, in this work I present the development and application of a plaque based approach for assaying malaria parasite growth in vitro. This method is very simple, and does not require expensive or complex equipment or extensive training. The plaque assay will therefore be an attractive and valuable approach within the malaria research community for analysing the effects of genetic mutation and drugs on parasite growth, including in resource poor settings.

Abstract

Malaria parasites invade erythrocytes and replicate inside a parasitophorous vacuole (PV). Invasive merozoites eventually egress in a process that involves sequential rupture of first the PV membrane (PVM) then the erythrocyte membrane. Egress is protease-dependent, with both cysteine and serine proteases implicated. The parasite serine protease SUB1 is stored in merozoite secretory organelles that are discharged into the PV ~10 minutes before erythrocyte membrane rupture. Pharmacological inhibition of SUB1 activity or discharge blocks egress, but the mechanism by which SUB1 regulates egress is unclear. In the PV, SUB1 cleaves multiple substrates including SERA6, a putative cysteine protease. In asexual blood stages of *Plasmodium falciparum*, the agent of the most dangerous form of malaria, *SERA6* is believed to be essential but its function and whether this depends on SUB1 is unknown. Here it is shown that conditional disruption of the *P. falciparum* *SUB1* or *SERA6* genes produces two distinct, lethal phenotypes. *SUB1*-null parasites undergo none of the morphological changes that precede egress and fail to rupture the PVM. In contrast, PVM rupture and the typical erythrocyte membrane poration occur normally in *SERA6*-null parasites but erythrocyte membrane rupture does not occur. Complementation studies demonstrate that SERA6 is an enzyme and that processing by SUB1 is required for its function. This study concludes that SUB1 and SERA6 play distinct, essential roles in a coordinated proteolytic cascade that enables sequential rupture of the two bounding membranes leading to egress.

Acknowledgements

I would like to express my gratitude to a number of people that have helped me greatly throughout my PhD project. Primarily I would like to thank and pay tribute to my supervisor Michael Blackman who supervised my PhD project. Amongst the parasitology research community Mike is both respected as a scientist and popular as an individual and his reputation played a great part in my decision to apply for a PhD project with him. Mike is an extremely insightful and meticulous scientist and has always been generous with his time and effort. I have learned a great deal from working with Mike and I try to emulate these characteristics myself. I would also like to thank all of the members of Mike's lab as well as those of Edgar Deu's lab who I have worked closely with during my time at the National Institute for Medical Research and now at the Francis Crick Institute (Nonlawat Boonyalai, Dara Annett, Sophie Ridewood, Emma Sherling, Abigail Perrin, Kostas Kousis, Mahsa Rahbari, Christine Lehmann, Asha Patel, Sujaan Das, Dafni Betchsi, Ross Hill and Christiaan van Ooij) for their friendship and professional help. I thank Helen Saibil and the members of her lab, Victoria Hale, Claudine Bisson and Trishant Umrekar as well as Roland Fleck and Gema Vizcay-Barrena for conducting electron microscopy work. In particular though I would like to thank Michele Ser Ying Tan, who has worked on a related aspect of *SERA6*. We have often combined our efforts in the lab, having at various times used the same cell lines and reagents. Chrislaine Withers-Martinez has always been ready to help me with my understanding of protease biochemistry and structural biology. Fiona Hackett and Christine Collins have helped with learning the ways of malaria parasite culture and Edgar Deu who has always provided extremely helpful suggestions, explanations and insights into my work.

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Chapter 1

Introduction

1.1 Malaria is caused by species of the genus *Plasmodium*

Malaria is a disease of humans caused by five species of the genus *Plasmodium*: *P. falciparum*, *P. vivax* [Battle et al. 2014; Kumari and Ghildiyal 2014], *P. knowlesi* [Singh et al. 2004; Cox-Singh et al. 2008], *P. ovale* [W. E. Collins and Jeffery 2005] and *P. malariae* [I. Mueller, Zimmerman, and Reeder 2007]. Recently however *P. ovale* has been demonstrated to actually consist of two non-recombining subspecies: *P. ovale curtisi* and *P. ovale wallikeri* [Sutherland et al. 2010]. *Plasmodium* is an obligate intracellular parasite is spread by the bite of *Anopheles* mosquitoes in tropical and subtropical regions throughout the world. In 2016, 91 countries reported a total of 216 million cases of malaria and approximately 445 000 deaths [WHO 2017]. Malaria incidence has fallen globally since 2010 but the rate of decline has stalled and in some regions has even reversed. Indeed 5 million more cases were reported in 2016 compared to 2015, although deaths remained about the same. The African regions accounts for about 90% of both cases and deaths globally. Throughout most of history the parasite had an even greater reach than it does now. *Plasmodium* belongs to the phylum Apicomplexa, a group of unicellular eukaryotes defined by the possession of an apical complex structure [Templeton et al. 2004] with most, including *Plasmodium*, possessing a plastid like organelle called an apicoplast [Kalanon and McFadden 2010]. The members of this phylum are exclusively endoparasites with the exception of *Nephromyces*, an endosymbiont of marine animals [Saffo et al. 2010]. The phylum contains various members which cause other diseases of medical and veterinary importance including toxoplasmosis in humans (*Toxoplasma gondii*) [Dubey 2009], coccidiosis in livestock, particularly poultry (*Eimeria spp*) [Macdonald et al. 2017], babesiosis in livestock (*Babesia spp*) [Westblade et al. 2017] and cryptosporidiosis in humans and livestock [Abrahamsen 2004].

Malaria parasites are ancient pathogens of humans and in one form or another have afflicted our ancestors from before our human lineage even diverged from that of our nearest living relatives, chimpanzees [Carter and Mendis 2002]. Malaria has undoubtedly had a profound impact on our recent evolution. Its effects are imprinted in the genetics of human populations across the globe, perhaps more so than for any other single infectious agent of disease [Carter and Mendis 2002]. Examples of this are the multiple genetic polymorphisms that contribute to innate immunity against the disease that have been selected

for during our evolution. These include: G6PD deficiency, various thalassaemias, sickle cell trait, haemoglobin C and E and ovalocytosis [Carter and Mendis 2002]. Duffy negativity has long been considered to render humans refractory to clinical infection with *P. vivax* [L. H. Miller, Mason, et al. 1975]. This trait has spread practically to fixation in West and Central African populations and this has been posited as the reason for the relative lack of *P. vivax* transmission from human populations in these regions [Bray 1958]. Relatively recently however cases of *P. vivax* infection, including clinical malaria, have been documented in Duffy-negative individuals using PCR based methods in Brazil [Cavasini et al. 2007; Carvalho et al. 2012], Ethiopia [Woldearegai, Kremsner, and Mordmu 2013; Lo et al. 2015], Madagascar [D. Ménard et al. 2010] as well as in the West African countries of Mauritania [Wurtz et al. 2011], Cameroon [Fru-cho et al. 2014; Mbenda and A. Das 2014], Equatorial Guinea and Angola [Mendes et al. 2011]. In Cameroon, it was found that 14.9% of malaria infections were caused exclusively or concomitantly by *P. vivax*, with 50% of those individuals infected with *P. vivax* being confirmed as Duffy negative by gene sequencing. In a different study a *P. vivax* specific ELISA using recombinant PvMSP1 (merozoite surface protein 1) and PvCSP1 (circumsporozoite protein 1) was developed as a cheaper and more scaleable alternative to molecular genetic methods for the screening of large populations. The performance of this ELISA was assessed in French travellers testing positive for *P. vivax* and French blood donors not previously exposed to *Plasmodium* [Poirier et al. 2016]. Following this validation with French subjects it was used to assess the proportion of 1,234 Beninese blood donors positive for *P. vivax*. Of the total 1,234 individuals tested 13.5% were only positive for antibodies against PvMSP1, 6.4% were only positive for antibodies against PvCSP1 and 15.2% were positive for antibodies against both PvMSP1 and PvCSP1. 84 individuals that were seropositive for antibodies against PvMSP1 and/or PvCSP1 were selected for further analysis using a nested-PCR approach to confirm infection with *Plasmodium* and determine which species were responsible for infection. Of 25 individuals that were positive by PCR for *Plasmodium* 13 were infected with *P. vivax* either exclusively or concomitantly with other *Plasmodium* species and all of these individuals were Duffy negative. These various studies clearly demonstrate that, contrary to the long held dogma, Duffy negativity does not constitute a complete barrier to infection with *P. vivax* and that the burden of *P. vivax* malaria is greater than previously appreciated in regions of West and Central Africa. The current epidemiological and treatment efforts are rightly focussed on *P. falciparum* but it will be important to consider the impact of *P. vivax*, especially in the context of elimination/eradication strategies. Clearly further work will be required to ascertain in exactly which populations *P. vivax* transmission occurs, and what mechanisms *P. vivax* uses to invade Duffy negative erythrocytes. It might be that some strains of *P. vivax* have evolved to use alternative blood groups for invasion. Alternatively some human populations might possess a unique blood group(s) that serves as a substitute for Duffy. Duffy negativity aside however, these relatively 'visible' effects of malaria selection pressure are probably just the tip of the iceberg when it comes to how malaria has shaped our biology and immune systems over evolutionary time [Kwiatkowski 2005].

For some time it was generally believed that *P. reichenowi*, a chimpanzee infecting species, was the closest living relative of *P. falciparum* (the most common cause of severe

malaria) and that the two species diverged concurrently with the divergence of the human and chimpanzee lineages [Prugnolle, Durand, et al. 2010]. However, it is now apparent that all available human infecting *P. falciparum* sequences constitute a single lineage that nests within a clade of western gorilla parasites [W. Liu et al. 2010]. This finding demonstrated that *P. falciparum* has arisen due to a host switch event from gorillas to humans. *P. falciparum* shows little genetic diversity at certain loci, which led to the suggestion that it had undergone a recent genetic sweep [Rich et al. 1998] but this may now be understood in the context of a host switch event leading to a genetic bottleneck. It remains unknown whether gorillas can act as reservoirs of *P. falciparum* that can then infect humans [W. Liu et al. 2010]. The occurrence of chloroquine-resistant *P. falciparum* (see Section 1.8.2) in chimpanzees clearly demonstrates a host switch from humans and these might be transmitted amongst chimpanzees and back to humans [Pacheco et al. 2013]. European travellers have returned home after visiting regions in West and Central Africa infected with *P. vivax*, which as discussed above is not generally transmitted amongst populations native to these regions due to the prevalence of Duffy negativity. This raises the intriguing possibility that *P. vivax*, or *P. vivax* like parasites, might be transferred zoonotically from apes or some other reservoir to humans, raising concerns regarding the control of disease [Prugnolle, Rougeron, et al. 2013]. As will be discussed *P. knowlesi* is now known to be transmitted zoonotically. Clearly the diversity of *Plasmodium* species amongst various primate hosts is great and host tropisms may be less well defined in reality compared to what was previously appreciated. Compounding this complexity is the fact that even if other primate species may harbour human infective malaria parasites, it is not inevitable that transmission to humans will occur. A host of biological and ecological factors may conspire against such an occurrence; infection of the animal may be transient, or parasitaemias low, gametocytes responsible for transmission may not form (see Section 1.3), or the mosquito vectors may not readily bite both species. An understanding of the diversity of malaria parasites and an assessment of these biological and ecological questions is therefore important for informing control strategies including those for local 'elimination' and global 'eradication'. Indeed, these uncertainties raises the somewhat troubling question: even if we do eliminate a human malaria parasite from a region or eradicate it from the globe, will another species just emerge to take its place?

1.2 Malaria remains a significant health burden

As discussed, the enormity of the burden of malaria throughout history is evident from the selective forces it has exerted on humankind. Malaria has partially or completely stymied a great many human endeavours, from the building of the Panama Canal to the advance of armies. The latter led British Army Colonel C H Melville to remark in Ronald Ross' book *The Prevention of Malaria* [Ross 1910] that 'the history of malaria in war might almost be taken to be the history of war itself'. Apart from the obvious morbidity and mortality of individuals, it also inflicts a tremendous socioeconomic burden [Malaney, Sielman, and Sachs 2004]. In endemic areas of Africa malaria predominantly afflicts children. When a child acquires malaria, they cannot attend school and their parents cannot work. With children suffering multiple clinical episodes a year in some regions the cumulative effects

of missed education and loss of adult productivity is a significant brake on non-health related quality of life and the economic prospects of society generally. While malaria has been successfully eliminated in Europe and North America, it continues to impose a great burden on the developing world [Sachs and Malaney 2002]. While this has been significantly reduced in the last decade by renewed efforts to control the disease, the disease still causes approximately 500,000 deaths per year. Ninety per cent of these are in Africa, where *P. falciparum* predominates and most are of children under five [WHO 2014]. It is also possible that future changes of climate and environment will lead to increases in malaria transmission in certain regions as well as emergence of the disease into previously transmission-free regions [Askling et al. 2012; Patz and Olson 2006].

While *P. falciparum* is considered the most virulent and deadly species causing malaria, there is an increasing appreciation of the importance of non-falciparum species as causes of morbidity and mortality [Val et al. 2017]. Due to a focus on falciparum malaria, basic research into the other species has been largely neglected and there is relatively little understanding of the epidemiology and biology of these species [Akerale et al. 2017; Bichara et al. 2017]. This is compounded by the inability to continuously culture the non-falciparum species *in vitro*, although *P. knowlesi* has recently been adapted to culture in human erythrocytes [Moon, J. Hall, et al. 2013; Lim et al. 2013]. Furthermore, other species are often disregarded or misdiagnosed in regions where falciparum malaria exists due to difficulties in distinguishing species by microscopy, masking their true impact [Mayxay et al. 2004]. *P. vivax* is capable of causing severe disease and death, particularly in children and pregnant women [Kumari and Ghildiyal 2014]. It also causes relapsing disease which will be an important consideration for elimination and global eradication efforts [Battle et al. 2014]. *P. knowlesi* is now known to routinely infect humans on the Malaysian peninsular and Borneo, often giving rise to severe and fatal disease characterised by hyper-parasitaemia [Ahmed et al. 2014; Singh et al. 2004; Cox-Singh et al. 2008]. New molecular screening techniques are demonstrating that the prevalence of *P. malariae* and *P. ovale*, generally assumed to be benign and rare, may have been underestimated [Ruas et al. 2017]. *P. malariae* and *P. ovale* may have important biological interactions with other species of *Plasmodium* such that control efforts specifically targeting and reducing the burden of *P. falciparum* and *P. vivax* might increase the incidence and burden of *P. malariae* and *P. ovale* [I. Mueller, Zimmerman, and Reeder 2007]. While eradication has reappeared on the global health agenda there remain significant obstacles to achieving this goal. These include emerging insecticide and drug resistance, increased human mobility, economic instability/underdevelopment, and inadequate tools to combat relapsing infection resulting from the dormant hypnozoite forms that can arise from *P. vivax* infection, not to mention the continued lack of an efficacious sterilising vaccine [White, Pukrittayakamee, et al. 2014; Battle et al. 2014; Sachs and Malaney 2002; Feachem et al. 2010].

1.3 *Plasmodium* lifecycle

Plasmodium, like all Apicomplexans, is predominantly an intracellular pathogen. The periods of intracellular existence within different cell types in the vertebrate and mosquito host are interspersed with extremely short periods of extracellular existence when the parasite

transitions from one cell to the next. Whilst outside of a cell, pathogens are maximally exposed to the host immune system. Therefore, *Plasmodium* likely faces selection pressure to limit the time it is exposed to the immune system to a minimum.

Infection of a vertebrate host with the pathogen begins when a female *Anopheles* mosquito takes a blood meal (Figure 1.1). In the process it injects ~10-100 sporozoite forms of the parasite [Frischknecht and Matuschewski 2017]. These migratory forms move within the dermis, apparently at random, until they contact a capillary [Amino, Thiberge, et al. 2006; Amino, Giovannini, et al. 2008]. They then traverse the capillary endothelium to enter the blood stream and travel to the liver where they traverse through the liver sinusoid endothelium and through several hepatocytes in an unusual process involving the breaching of the plasma membrane followed by rapid repair with no formation of a parasitophorous vacuole (PV) [Mota et al. 2001]. Eventually they invade a hepatocyte in a process that results in formation of a parasitophorous vacuole, in which they reside [Risco-Castillo et al. 2015]. Sporozoites use the higher sulfation level of host heparan sulfate proteoglycans on hepatocytes as a signal to invade a hepatocyte [Coppi et al. 2007]. Over the ensuing 5-10 days, repeated rounds of non-synchronous nuclear replication within this vacuole (termed schizogony) results in a multi-nuclear syncytium called a schizont. Budding of the parasite plasma membrane around the nuclei results in several thousand hepatic merozoite forms. The parasites then rupture the parasitophorous vacuole membrane (PVM) and induce the death and detachment of the infected hepatocyte from neighbouring cells. The merozoites are released into the blood stream within host-derived vesicles that bud off from the hepatocyte, called merosomes [Sturm 2006]. Merosomes extrude into the lumen of the liver sinusoid where they flow to the pulmonary capillaries. The parasites appear to suppress the exposure of phosphatidylserine on the outer leaflet of the host membrane of merosomes which normally acts as a signal for phagocytes to phagocytose a cell [Friedrich et al. 2012a]. As merozoites are readily phagocytosed by the abundant Kupffer cells in the liver sinusoid, merosomes likely ensure the survival of merozoites until they have exited the liver. The merosomes eventually lodge in pulmonary capillaries and merozoites are released. It has been suggested that this is an advantageous site for merozoite release due to a low macrophage density and reduced blood velocity which reduces shear forces, allowing the merozoites to invade erythrocytes more successfully [Baer et al. 2007].

The invasion of erythrocytes initiates the asexual blood stage cycle. During this stage repeated rounds of invasion, replication of daughter merozoites and egress result in exponential proliferation of parasites. It is this stage, with the lytic destruction of erythrocytes during egress, that results in all the clinical symptoms of malaria [White, Pukrittayakamee, et al. 2014]. Due to its clinical relevance and the ease with which it can be cultured continuously *in vitro*, the asexual blood stage cycle has been the most studied. Invasion of an erythrocyte begins with initial attachment of the merozoite to the surface of an erythrocyte (Figure 1.2) [Cowman et al. 2017; Tham et al. 2015]. The merozoite then induces waves of deformation across the erythrocyte before it reorients such that the apical complex is in contact with the erythrocyte surface. The merozoite then invades the erythrocyte by apparently pushing its way into the cell, with force generated by an actinomyosin motor [Cowman et al. 2017]. This involves the formation of a 'moving junction' which was first observed as an electron dense interface at the point of contact between the merozoite

and erythrocyte membranes in electron microscopy studies over 30 years ago [Aikawa, L. H. Miller, and J. Johnson 1978]. The moving junction is known to be formed via interactions between apical membrane antigen 1 (AMA1), an integral protein secreted onto the merozoite surface from specialised parasite secretory organelles called micronemes and a complex of RON (rhoptry neck-derived) proteins secreted into the erythrocyte from another type of specialised secretory organelles called rhoptries [Richard et al. 2010]. Invasion culminates in the resealing of the erythrocyte behind the merozoite and the budding off of the invaginated erythrocyte membrane to form the parasitophorous vacuole membrane (PVM) [Paul, Egan, and Duraisingh 2015; Cowman et al. 2017]. For the first ~14 hours post invasion the parasite resembles a diamond ring in Giemsa stained thin films, giving rise to the name ring stage. This develops into a trophozoite (feeding) stage, which digests haemoglobin from the erythrocyte, resulting in the formation of a food vacuole that contains crystallised haem called haemozoin. Eventually the parasite undergoes DNA replication by schizogony as in the liver stage. Multiple rounds of asynchronous nuclear division results in a syncytium containing 20-25 nuclei, followed by apportioning of organelles to each nucleus and budding of the parasite plasma membrane around each nucleus to form daughter merozoites. A by-product of this is the formation of a 'residual body' consisting of the food vacuole surrounded by membrane. Egress of these merozoites (discussed in more detail in Section 1.13) allows the invasion of fresh erythrocytes and initiation of the next growth cycle.

Sexual precursor forms called gametocytes are the forms required for transmission to mosquitos. Their development forms an offshoot of the asexual blood stage cycle. This switch into either male or female gametocyte development appears to be regulated by a member of the ApiAP2 family of transcription factors [Lindner et al. 2010]. When this switch takes place all of the merozoite progeny of a single parasite are destined to develop into gametocytes of the same sex [Bruce et al. 1990; Inselburg 1983; Williams 1999]. Most gametocytes of most *Plasmodium* species reach maturity in two days, however in *P. falciparum* the process takes about 7-10 days [Bousema and Drakeley 2011]. Upon entering a mosquito midgut, mature gametocytes rapidly activate and transform into gametes in response to a combination of environmental stimuli, including a lower temperature compared to the mammalian host and the presence of the tryptophan metabolite xanthurenic acid which is present at relatively high levels in the insect midgut [Billker et al. 1998; Chevalley et al. 2010]. The gametes then undergo egress from the human erythrocyte, with male gametes first undergoing three rounds of DNA replication to become octaploid. Eight flagellated microgametes result which detach from the residual body of the cell. These fertilise the female macrogametes to form a zygote which over 20 h develops into an ookinete. This moves by gliding motility through the midgut wall where it forms an oocyst embedded in the basal lamina of the mosquito midgut. This eventually ruptures, releasing ~3000 sporozoites [Rosenberg and Rungsiwongse 1991; Frischknecht and Matuschewski 2017] which migrate through the haemolymph to the salivary glands and undergo maturation. Little is known about this maturation process but the proteomes of salivary gland and midgut sporozoites differ quite markedly [Lasonder et al. 2008]. Eventually these mature salivary gland sporozoites are transmitted back into a human host when a mosquito takes a blood meal.

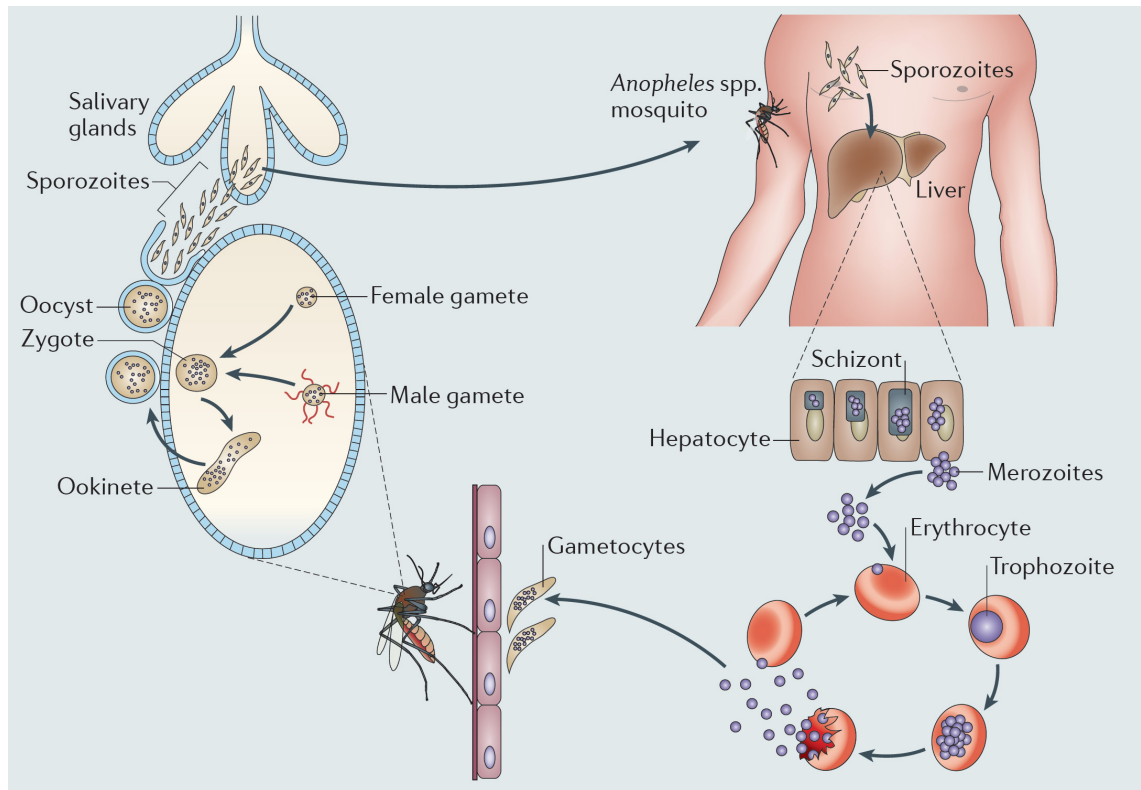


Figure 1.1: **The *P. falciparum* life cycle.** The life cycle of *Plasmodium* is divided between the *Anopheles* mosquito vector and the vertebrate host. Infection of a human is established by injection of sporozoite forms of the parasites from the mosquito into the skin as the mosquito takes a blood meal. The sporozoites migrate to the liver and invade hepatocytes. The parasite replicates by schizogony to form daughter merozoites which are released into the bloodstream where they invade erythrocytes, establishing the asexual blood stage cycle. During the asexual blood stage cycle, repeated rounds of invasion, replication and egress propagates the parasite population exponentially. This stage of the life cycle gives rise to all the clinical symptoms of malaria. Some merozoites that invade fresh erythrocytes develop into male or female gametocytes which are the sexual forms responsible for transmission back into a mosquito. When a blood meal is taken the gametocytes undergo gametogenesis in the mosquito midgut to form gametes which egress from the confines of the erythrocyte. The male gametes fertilise the female gametes to form a zygote which develops into an ookinete. Ookinetes glide through the midgut, traverse through the midgut wall and embed within the basal lamina to develop into an oocyst. In the oocyst, many sporozoites form which are eventually released and migrate to the salivary glands ready to infect a new host when the mosquito takes a blood meal. From [Koning-Ward, Gilson, and Crabb 2015]

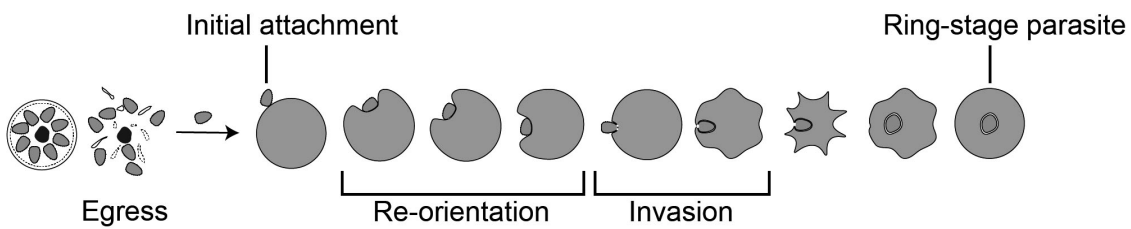


Figure 1.2: **Merozoite invasion of an erythrocyte.** Invasion of an erythrocyte begins with initial attachment of the merozoite to the surface of the erythrocyte and may occur in any orientation. The merozoite then deforms the erythrocyte as it reorients such that the apical end is in contact with the erythrocyte. The merozoite then pushes into the cell using the force of its actin myosin motor, before the erythrocyte membrane pinches off to reseal on the outside and form the PV on the inside. Echinocytosis, whereby the erythrocyte membrane becomes spiky in appearance, occurs transiently before the erythrocyte returns to its normal state. Adapted from [Yap et al. 2014].

1.4 All of the pathology of malaria derives from the asexual blood stage cycle

As mentioned above, malaria infection is initiated by invasion of liver cells. Clinical symptoms of malaria only manifest following establishment of the asexual blood stage infection however. In cases of uncomplicated malaria these include fever, chills, nausea, headache, vomiting, and diarrhoea. These symptoms are believed to be caused by tumour necrosis factor, the release of which is stimulated by production of malaria 'endotoxin' from parasites, which is understood to be a complex of haemozoin and parasite DNA [Wijesekera et al. 1996; Karunaweera et al. 1992]. Anaemia results from rupture of erythrocytes as well as dyserythropoiesis that occurs during infection [Kwiatkowski 2005; L. H. Miller, Ackerman, et al. 2013; Pathak and Ghosh 2016].

Severe malaria is usually caused by *P. falciparum* but can also be caused by *P. vivax* and *P. knowlesi* and involves complications including severe anaemia, coma, pulmonary oedema, hypoglycaemia and acute kidney injury [Phillips et al. 2017]. Severe malaria occurs in 1% of African children and kills 10% of those afflicted and although less common in adults it kills 20% of those afflicted. Severe malaria is more common in individuals with reduced immune protection such as those in low transmission settings [Phillips et al. 2017]. The most susceptible however are children between 6 months and 5 years of age. At this stage they have lost their maternal antibodies but not yet developed immunity themselves [Phillips et al. 2017]. In addition, co-infection with various pathogens is common in malaria endemic regions including HIV, *Mycobacterium tuberculosis* and helminths. HIV-infection increases the risk of severe malaria and death and infection with other pathogens may be associated with adverse outcomes [Hochman and K. Kim 2009]. Our understanding of the pathophysiology and mechanisms of severe malaria remain far from complete and what causes some individuals to become afflicted by severe forms of the disease while most individuals suffer relatively benign uncomplicated disease is unknown [Wassmer et al. 2015]. A major source of pathology during severe malaria infection is the sequestration of infected erythrocytes to the vascular endothelium which results in the avoidance of parasite clearance by the spleen. Endothelial adherence is achieved through export of PfEMP1 (erythrocyte membrane protein-1) onto the surface of infected erythrocytes which mediates binding to endothelia via interactions between endothelial protein C receptor (EPCR), the endothelial surface marker cluster of differentiation (CD) 36 and intercellular adhesion molecule-1 (ICAM-1) [Maier et al. 2009; Tuikue Ndam et al. 2017]. The sequestration of infected erythrocytes damages endothelial cells as well as disrupting blood flow, causing tissue hypoxia and tissue acidosis [Brand et al. 2016]. Furthermore, the haemolysis of infected and bystander erythrocytes leads to anaemia which may be compounded by reduced erythropoiesis [Pathak and Ghosh 2016]. Haemolysis also exacerbates endothelial damage, with free haemoglobin catalysing production of reactive oxygen species, which results in oxidative damage to cells [Schaer et al. 2013]. These mechanisms are also responsible for organ specific syndromes associated with severe malaria, such as cerebral malaria and placental malaria, when sequestration occurs in the brain or placenta respectively. Cerebral malaria is a life-threatening event causing the death of 15-20% of those afflicted and ~25% develop long-term neurological sequelae.

A magnetic resonance imaging study of cerebral malaria patients in India demonstrated that brain swelling mainly results from loss of brain endothelial integrity [Mohanaty et al. 2017] potentially revealing a mechanism for a symptom of severe malaria that is strongly associated with death [Gallego-Delgado and Rodriguez 2017]. This is supported by the finding that rupture of infected erythrocytes over human brain endothelial cell monolayers *in vitro* resulted in the opening of intercellular gaps [Gallego-Delgado and Rodriguez 2017].

Placental malaria most commonly occurs in Africa, caused by *P. falciparum*, but also occurs outside of Africa, where it is generally caused by *P. vivax* [Howes et al. 2016]. Placental malaria often leads to poor outcomes for foetus and mother. Pregnancy increases a mother's susceptibility to infection, especially during their first pregnancy, as they will have yet to acquire immunity to parasites expressing the variant surface antigen VAR2CSA on the erythrocyte surface which binds to placental chondroitin sulfate A (CSA) and causes sequestration in the placenta [Pereira et al. 2016]. The risk of placental malaria is reduced in multigravid women from endemic areas who generally possess antibodies against this antigen. Placental malaria can be asymptomatic or mild but leads to an increased risk of death for both baby and mother and predisposes the mother to miscarriage and stillbirth. It also increases the chance of premature delivery or delivery of babies with low birth rate with the subsequent reductions in survival and quality of life that these events bring [Phillips et al. 2017].

1.5 Antimalarial vaccines

Vaccines are the most efficacious and cost-effective means of combatting disease, and have been developed to prevent a number of bacterial and viral diseases including rabies, smallpox, polio, bacterial meningitis, typhoid and diphtheria to name a few. Mass vaccination strategies have eradicated smallpox [Okwo-Bele and Cherian 2011; Metzger, Kohler, and Mordmuller 2015] and eliminated polio from all but a few regions of the world [Kew et al. 2005; Morales, Tangermann, and Wassilak 2016]. A great benefit of vaccines is that they prevent the occurrence of disease in the first place, allowing transmission cycles to be broken which greatly facilitates elimination/eradication strategies. An efficacious sterilising vaccine against malaria would greatly facilitate regional elimination and even global eradication of the disease. All of the widely implemented and efficacious vaccines developed thus far have been for bacterial and viral infections that the immune system is capable of generating sterilising immunity to naturally. As such, many vaccines effectively stimulate the immune system into developing immunity by inoculation with inactivated or attenuated infectious agents, as if during a natural infection, and have often been successful in doing so with little to no prior knowledge of the mechanisms of immunity generated to these pathogens [Sette and Fikes 2003].

In the case of malaria, while immunity does develop in people in areas of endemic transmission, it is not sterilising, and protection wanes over time in the absence of continued exposure to infection [Doolan, Dobaño, and Kevin Baird 2009]. The malaria parasite is also antigenically diverse. Such diversity in protective antigens means achieving cross-strain protection from antibodies is very difficult. Such diversity has also hampered the

development of vaccines to HIV, *Streptococcus pneumoniae*, Hepatitis C virus and influenza virus [Nickle et al. 2007; Takala and Plowe 2009]. Compounding this problem for the malaria parasite is that it is a highly immune-evasive organism with various antigenically distinct stages [Schwartz et al. 2012]. Perhaps because of this, the majority of the considerable number of attempts made to develop a vaccine against malaria have not proved widely effective or practical [Schwartz et al. 2012; R. Ménard 2005]. It has long been known that irradiated sporozoites are capable of consistently generating sterilising immunity against malaria infection in a range of hosts. This was first demonstrated in 1941 for a form of avian malaria [P. Russell, Mulligan, and Mohan 1941] and has since been demonstrated in humans, including cross strain immunity [Clyde et al. 1973; Hoffman, Goh, et al. 2002]. This protection required that volunteers be bitten by a large number of irradiated infected mosquitos (more than 1000) however, which is entirely impractical for clinical application. Even then immunity waned within a year or two. Another study has also demonstrated that multiple immunisations with sporozoites by mosquito bite while receiving chloroquine prophylaxis produced antibodies that were capable of disrupting sporozoite traversal in the liver [Behet et al. 2014].

The impractical nature of whole cell sporozoite inoculations by mosquito bite or otherwise, along with concerns that safety might be difficult to ensure, has led to an interest in generating protein based vaccines. A significant number of trials have been conducted, mostly using blood stage antigens including: AMA1, MSP1, MSP3, SERA5, EBA175, RESA and GLURP [Schwartz et al. 2012]. To date the most advanced vaccine of any kind is the RTS,S/AS01 recombinant protein based vaccine developed by GSK which is a formulation containing a fragment of the sporozoite surface protein CSP [Multiple 2012; Casares, Brumeanu, and Richie 2010]. In an 18 month phase III clinical trial RTS,S/AS01 was found to reduce the incidence of malaria by about 50% in young children, but did not reduce mortality rates of those with clinical disease. Nevertheless it must not be forgotten that this is not only the first ever licensed vaccine for malaria, but the first for any parasitic disease. Despite the shortcomings of RTS,S/AS01, it has demonstrated that it is possible to impact the host-parasite interaction during infection through vaccine induced immune responses to antigenic subunits [Schwartz et al. 2012]. It has also been shown that antibodies generated in mice by inoculation with RTS,S/AS01 in one animal can provide sterilising immunity against *P. berghei* when antibodies are transferred to another animal [Foquet et al. 2013] raising the possibility that this vaccine might serve as a foundation from which to develop a more efficacious vaccine. Preliminary data from a recent study has suggested that optimisation of the immunisation regimen may significantly increase the protection offered by the RTS,S/AS01 vaccine [Regules et al. 2016]. RTS,S/AS01 is due to be rolled out in three countries in Africa in 2018 in a WHO coordinated programme to assess the effects and feasibility of the vaccine (which requires 4 doses) in the context of routine use. The malaria and broader public health communities will no doubt come up with creative ways of making the best of even this imperfect vaccine. Highly targeted administration based on predictions of seasonal transmission or for regional elimination has been suggested for example [Greenwood, Dicko, et al. 2017]. Nevertheless some significant improvements will be required before we have a vaccine with the potential to achieve global eradication and this will likely require a better understanding of parasite

biology and how the host interacts with the parasite during infection.

1.6 Current priorities and strategies for vaccine development

Potential malaria vaccines can be grouped into three broad categories. Those that act on clinically silent pre-erythrocytic stages to prevent establishment of clinically relevant asexual blood stage infection, those that act on the erythrocytic stages to facilitate parasite clearance once they progress to the erythrocytic stages and those that prevent onwards transmission from an infected human. The latter would be expected to provide herd immunity which would reduce malaria incidence [Hoffman, Vekemans, et al. 2015]. An ideal malaria vaccine requires three features. The first is multiple components that induce an immune response against sporozoites, infected hepatocytes and asexual and sexual blood stages. The second is multiple epitopes that are restricted to presentation by different major histocompatibility complex molecules to overcome genetic diversity and antigenic variation. Thirdly, it should have multi-immunogenicity including more than one type of immune response including cell-mediated and humoral responses to increase the chances of achieving a sustainable and effective host-response [Arama and Troye-Blomberg 2014]. New vaccine candidates being pursued include PfRh5, an essential erythrocyte invasion ligand of the parasite. PfRh5 has been shown to induce protective antibodies that are effective across common genetic variants of PfRh5 [Bustamante et al. 2013; Douglas et al. 2011]. As discussed in Section 1.4 VAR2CSA, a member of the PfEMP1 variant surface antigen family, is responsible for sequestration of *P. falciparum* in placenta. With multiple pregnancies women produce antibodies against VAR2CSA and these antibodies are associated with increased birth weights and better survival and outcomes for their offspring [Fried and Duffy 2015]. Efforts are underway to develop a subunit vaccine which will stimulate the production of protective anti-VAR2CSA antibodies with two VAR2CSA based products expected to enter clinical trials in the near future [Fried and Duffy 2015].

In addition to these promising subunit vaccines attenuated vaccines also continue to be developed. The Sanaria company was established to translate the mosquito based immunisation approach discussed above into an injectable whole sporozoite malaria vaccine to eliminate malaria from specific regions and protect non-immune travellers. Sanaria is engaged in developing an injectable attenuated sporozoite vaccine that is aseptic, pure, adequately attenuated and potent. Sanaria has demonstrated that it is possible to achieve protection by intravenous injection of sporozoites cryopreserved in liquid nitrogen vapour phase which obviates the requirement of electricity for cryopreservation [Seder et al. 2013]. Sanaria is now moving to scale up manufacturing, suitable for a phase 3 clinical trial [Hoffman, Vekemans, et al. 2015] but for the time being at least this remains far from practical for widespread use in the clinic given the difficulty in obtaining sporozoite material in sufficient quantities and the number of doses (more than four doses) required to achieve protection.

Genetic attenuation is another means by which attenuated whole parasite vaccines might be developed. With the recent improvements in genetic approaches discussed in Section 1.11 it is now possible to embark on rational and directed attempts to develop

genetically attenuated parasites for use in vaccines. It has been shown that knockout of the *UIS3* (up-regulated in sporozoites 3) gene of *P. berghei* results in parasites that infect hepatocytes but fail to initiate blood-stage infection and do not lead to disease. Crucially immunisation with *UIS3* parasites provided complete protection against challenge with sporozoites [A.-K. Mueller, Labaied, et al. 2005]. Similar findings were seen with knockout of *UIS4* (up-regulated in sporozoites 4) in *P. berghei* [A.-K. Mueller, Camargo, et al. 2005]. This approach has also been used to produce genetically attenuated *P. falciparum* sporozoites by disruption of p52 [Schaijk et al. 2008]. A recent study generated genetically attenuated *P. falciparum* sporozoites by deletion of three genes, simultaneously: p52, p36 and sap1 [Kublin, Mikolajczak, et al. 2017]. These parasites were delivered to 10 volunteers by means of mosquito bite and all individuals remained free of blood-stage parasites and developed inhibitory antibodies to sporozoites. This is an important proof of principle as ensuring sufficient attenuation of parasites, whilst retaining immunogenicity may be difficult to ensure [Vaughan et al. 2017].

1.7 Control strategies against malaria

In the absence of an efficacious vaccine a wide range of different measures and strategies have been used to control malaria, targeting both the mosquito vector and parasite itself. There has long been an interest in controlling mosquito populations given their role as vectors of various diseases, including malaria. Historically, destruction of mosquito breeding grounds has been a widely implemented and successful strategy for the reduction of mosquito populations. In the early 20th century this consisted of draining swamps and emptying, covering or oiling pools of standing water. As an example, such strategies were implemented by Dr William Gorgas, an American Army surgeon in the Panama Canal Zone, which resulted in an 80% decrease in cases of malaria amongst canal company employees [Chapin and Wasserstrom 1981]. Such measures helped achieve reduction of malaria cases in Southern Europe and the United States of America alongside general improvements in sanitation and the use of window screens and bed nets.

Insecticides have proved to be the most successful tool for controlling mosquito populations. The best known of several chlorine containing pesticides used in the 1940s and 50s was DDT (dichlorodiphenyltrichloroethane). Malaria was a great health concern for the Allies during the second world war, with considerable numbers of troops debilitated or killed by the disease. Aerial spraying of DDT was used with considerable success in the South Pacific against mosquitos carrying malaria and dengue. Starting between 1952 and 1955, the World Health Organisation embarked on a programme to eradicate malaria with great confidence [Hay et al. 2004]. This programme achieved considerable success. For example, in Sri Lanka the programme reduced the approximately one million cases per year to just 18 by 1963. However, failure to sustain the programme due to economic concerns and political instability as well as the development of behavioural and physiological resistance of mosquitos to DDT, plus concerns about wider environmental effects of DDT, caused the partial or even total reversal of progress in many regions [Sougoufara et al. 2017]. In fact, in Central America, where measures were implemented in the late 1960s the incidence of disease was three times higher following the cessation of control

efforts in 1975 than a decade earlier [Chapin and Wasserstrom 1981]. Nevertheless, in countries with low to moderate transmission rates and well organised health care systems to rapidly diagnose and treat patients, the transmission cycle was broken and elimination was achieved in 37 countries including Taiwan, most of the Caribbean, the Balkans, parts of North Africa, Northern Australia and large parts of the South Pacific [T. L. Russell et al. 2013].

A diverse range of mosquito control strategies continue to be investigated for the control of a range of mosquito species. This is not just limited to the malaria carrying Anopheleline mosquitos but also for mosquito species of the genus Aedes that carry Dengue fever and other diseases [Benelli and Beier 2017; Macias, Ohm, and Rasgon 2017]. These include architectural solutions such as ‘eve tubes’ in houses that suck mosquitoes into insecticide treated nets with fans, insecticide treated clothing, biocontrol through the introduction of aquatic predators of mosquito larvae and treatment of humans with drugs that kill mosquitos upon taking blood meals [Benelli and Beier 2017]. Another strategy which continues to attract much interest is the sterile insect technique (SIT). A method of releasing male screwworms (*Cochliomya hominivorax*) that were sterilised by exposure to X-rays led to the elimination of this agricultural pest in North America in 1968 [Bouyer and Lefrançois 2014]. A similar approach was successful in eradicating a species of tsetse fly (*Glossina austeni*), which transmits trypanosomiasis, from Unguja Island in Zanzibar [Macias, Ohm, and Rasgon 2017]. This success formed the basis for modern attempts to release genetically engineered sterile mosquitoes. It has also been proposed to combine SIT with another technique, auto-dissemination whereby wild mosquitos are contaminated with juvenile hormone in dissemination stations which prevents the maturation of larvae [Bouyer and Lefrançois 2014]. SIT has achieved promising successes but significant challenges remain before this approach will allow region-wide, let alone continent-wide, control [Oliva et al. 2012].

In the absence of a highly efficacious vaccine, current malaria control relies on three major interventions: insecticide treated bed nets (ITNs), artemisinin combination therapies (ACTs) (as well as other drug treatments) and indoor residual insecticide spraying. The scaling up of these have been highly successful in reducing the burden of disease; between 2000 and 2015 the global incidence of malaria fell by 37% and malaria-related mortality by 60%. It has been estimated that 663 million clinical cases of malaria have been averted due to interventions during this period, with 69% of the reduction in disease incidence and mortality attributable to ITNs, 21% to ACTs and 10% to insecticide spraying [Bhatt et al. 2015].

1.8 Antimalarial chemotherapy

Various drugs exist for the treatment of malaria. Providing they are administered promptly these can generally be relied upon to provide favourable outcomes with cure rates of 98% [Phillips et al. 2017]. The drugs all act by killing blood stage parasites, which cause the clinical symptoms of malaria, to reduce parasite burden and its associated pathology [L. H. Miller, Ackerman, et al. 2013]. Despite this, lack of availability, high costs, and emergence of resistance continue to hamper the effective use of chemotherapies against malaria on

a global scale. Artemisinin based combination therapies (ACTs) are the recommended first line therapies for uncomplicated falciparum malaria in all endemic regions [Visser, Vugt, and Grobusch 2014] (discussed in Section 1.8.6). These combine a highly efficacious artemisinin derivative with another antimalarial that is slowly eliminated from the body in order to guard against resistance to the former, which has a short half-life [Kavish, Koenderink, and Alifrangis 2017]. ACTs are highly efficacious against all forms of human malaria and are increasingly considered for treatment of non-falciparum malaria. However clinical resistance to ACTs is beginning to emerge [Docherty et al. 2003]. In western Cambodia and the Myanmar-Thailand border regions, the same regions where chloroquine and sulfadoxine-pyrimethamine resistance emerged, reduced rates of parasite clearance are being observed [Docherty et al. 2003; Arie et al. 2014]. This is a grave threat to control strategies, with the potential to undo much of the progress made in reducing malaria morbidity and mortality over the last decade. This makes it imperative to stoke the development pipeline and increase our understanding of parasite biology. There is also the need for drugs which can target the non-erythrocytic hypnozoite stage of *P. vivax* to provide ‘radical cure’ and prevent the existence of latent carriers of disease [Markus 2010; Battle et al. 2014; Wells, Burrows, and Baird 2010]. Currently the only drug available that targets hypnozoites is primaquine and related 8-aminoquinolines, which are contraindicated in G6PD deficient patients [Delves et al. 2012]. This is particularly problematic given that G6PD deficiency is prevalent amongst populations in regions of malaria transmission precisely because it has been selected for by providing some level of protection against malaria (see Section 1.1). What follows in this section is a brief summary of some of the key antimalarials that have been and continue to be used.

1.8.1 Quinine

Quinine is the first drug that was ever used to treat malaria infection, and in fact, the first chemotherapeutic agent used to treat any infectious disease. From the 1600s onwards the quinine-containing bark of the cinchona tree was consumed for the treatment of febrile ailments including malaria. In 1820 quinine was extracted from the bark by Pierre Joseph Pelletier and Joseph Caventou and this replaced the crude extracts as the standard treatment for malaria. In fact, multiple alkaloids from cinchona bark were found to be effective at causing the ‘cessation of febrile paroxysms’ [Achan et al. 2011] but quinine became the primary alkaloid used (Figure 1.3). Quinine contains a quinoline group, various derivatives of which have been developed and deployed as antimalarials. Quinine remained the predominant treatment for malaria until the 1920s when the more effective synthetic derivative of quinoline, chloroquine, became available [Achan et al. 2011]. Chloroquine was used particularly heavily during the Second World War when a great many troops were exposed to the disease in the South East Asian, Pacific and North African theatres of war. Resistance began to be seen in Southeast Asia and South America by the late 1950s and became widespread by the 1980s. With resistance to chloroquine, quinine became an important treatment for malaria once again. Quinine continues to be an important drug for the management of malaria infection despite significant drawbacks to its therapeutic use; side-effects are numerous and can be severe, and treatment regimens are extended (five to seven days) which hinders compliance [Achan et al. 2011]. Quinine in combination

with doxycycline, tetracycline or clindamycin is recommended as second-line treatment for uncomplicated malaria upon failure or unavailability of first-line treatment. Additionally, even in cases where ACTs should be preferred, a lack of availability as well as the cost of ACTs means quinine is often administered in Sub-Saharan Africa where it thus remains a mainstay of malaria treatment. Interestingly despite the centuries of use, emergence of resistance to quinine has been slow, and usually low-grade. It retains activity with resistance generally extending treatment times rather than causing outright failures. This is in contrast to resistance against chloroquine. This means that about 400 years later quinine retains its place in our arsenal of antimalarials. Furthermore the gametocidal activity of quinine holds potential to develop quinine or related quinoline derivatives as transmission blocking agents for control and elimination/eradication strategies.

1.8.2 Chloroquine

As mentioned above, chloroquine was developed as a synthetic quinoline derivative (Figure 1.3). It was found to be highly efficacious, it exhibited low toxicity and is cheap to manufacture [Greenwood, Bojang, et al. 2005]. It was therefore recommended for the prophylaxis and treatment of all malaria infections and related compounds were developed. Chloroquine sequesters in the food vacuole and is believed to act by interfering with haemoglobin digestion by the parasite [Slater 1993]. During the WHO malaria eradication programme of the 1950s and 1960s (see Section 1.7) vast amounts of chloroquine were administered worldwide. As such, parasite populations were subject to substantial selection pressures and resistance emerged, first in South America in 1959 and then independently in Cambodia in 1960 [Slater 1993]. Chloroquine eventually failed almost everywhere for the treatment of falciparum malaria [Greenwood, Bojang, et al. 2005] as well as for vivax malaria [Baird 2004; Tjitra et al. 2008]. Resistance against chloroquine has been associated with lower concentrations of chloroquine in the food vacuole and has been linked to a transporter in the food vacuole called chloroquine resistance transporter (pfcrt) [Fidock, Nomura, et al. 2000]. Interestingly however there have been a number of reports of increases in chloroquine susceptibility since its removal from front-line use and loss of the associated selection pressure [Kublin, Dzinjalama, et al. 2002; Mwai et al. 2009; Gharbi et al. 2013]. Chloroquine has a good safety profile and is cost-effective. The high costs of artemisinin derivatives and the emergence of resistance to them mean novel derivatives of chloroquine remain an attractive prospect [Aguiar et al. 2012].

1.8.3 Sulfadoxine-pyrimethamine

Sulfadoxine and pyrimethamine are antifolate drugs, so called as they interfere with the parasite's folate biogenesis pathway through inhibition of dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR) respectively [Ngondi et al. 2017]. The drugs act synergistically and in the face of widespread treatment failures to chloroquine across the world sulfadoxine-pyrimethamine combination therapy (SP) was rolled out in the 1990s. In 1993, Malawi became the first African country to recommend it as first-line therapy, and many African nations followed suit soon after [C. H. Sibley et al. 2001]. Resistance emerged in Southeast Asia and South America exceptionally rapidly however. As such the useful ther-

apeutic lifespan of SP was less than five years [C. H. Sibley et al. 2001]. Interestingly this resistance emerged out of the same region, Cambodia, from which chloroquine resistance emerged. Resistance to SP has been shown to result from the ordered accumulation of point mutations in the DHPS and DHFR genes, as well as copy number increases of these mutant alleles [Kublin, Dzinjalama, et al. 2002; C. H. Sibley et al. 2001; Ngondi et al. 2017]. Despite this, the WHO continues to recommend SP (due to its safety) for intermittent preventative treatment in pregnancy in Africa to reduce the burden of adverse effects that afflict malaria infected pregnant women and their fetuses or neonates [Ngondi et al. 2017].

1.8.4 Mefloquine

The antimalarial properties of Mefloquine were discovered as part of the Walter Reed Army Institute of Research malaria drug discovery programme which ran from 1963-1975; the largest drug discovery programme ever mounted at the time [Croft 2007]. Mefloquine became available for prophylactic use in Europe in 1985 and in the USA in 1990 [Hagmann 2017]. In the face of widespread chloroquine resistance, at a time when international travel was increasing exponentially, mefloquine became one of the main drugs recommended for chemoprophylaxis [Schlagenhauf, Adamcova, Regep, Schaerer, and Rhein 2010]. Importantly mefloquine is sanctioned by the WHO for use during the second and third trimesters, and even in the first trimester by some authorities [Schlagenhauf, Adamcova, Regep, Schaerer, and Rhein 2010; González et al. 2014]. It is also considered safe during breast-feeding [Schlagenhauf, Adamcova, Regep, Schaerer, and Rhein 2010]. Mefloquine was (and still is) considered effective against the blood stages of all human malaria species, including *P. knowlesi*, apart from in clearly defined regions of multi-drug resistance [Schlagenhauf, Adamcova, Regep, Schaerer, and Rhein 2010]. The safety of Mefloquine has been the subject of some considerable controversy. In particular it has allegedly been associated with adverse psychiatric events including suicide [Croft 2007]. However a recent Cochrane review of more than 50 randomised and non-randomised studies with more than 1 million patients using mefloquine to prevent malaria found serious side-effects were not more frequently seen than with atovaquone-proguanil or doxycycline [Tickell-Painter et al. 2017]. Given the concerns over resistance to ACTs there is a desire to have as many different combinations of drugs available as possible. As such mefloquine, with its long half life of elimination, is an important partner drug for use with the artemisinin derivative artesunate [Schlagenhauf, Adamcova, Regep, Schaerer, Bansod, et al. 2011] (discussed in Section 1.8.6). Artesunate-mefloquine combination therapy is not widely used in Africa and is even unregistered in many African nations despite being recommended by the WHO for treatment of uncomplicated malaria [Eziefula 2016]. Artesunate-mefloquine has been shown to be as effective as artemether-lumefantrine, the most widely used ACT in Africa, and safe in children so should become a valuable treatment option [Schlagenhauf, Adamcova, Regep, Schaerer, Bansod, et al. 2011; Sirima et al. 2016].

1.8.5 Piperaquine

Piperaquine was synthesised independently by both the Shanghai Pharmaceutical Industry Research Institute in China and Rhone Poulenc in France in the 1960s. It was found to be potent against chloroquine resistant parasites and had good tolerability so in the face of widespread chloroquine resistance it was recommended by the Chinese National Malaria Control Programme in 1978 [Davis et al. 2005]. Widespread unregulated use as monotherapy throughout China since the late 1970s appears to have played a significant role in the development of resistance to piperaquine. Piperaquine is now used in combination with dihydroartemisinin (DHA) due to its long half-life, favourable safety and toxicity profile and relatively low cost, the latter making DHA-piperaquine co-formulations attractive for resource poor settings [Davis et al. 2005].

1.8.6 Artemisinin combination therapy

In the face of widespread resistance to the antimalarials outlined above a new antimalarial chemotherapy was desperately needed by the late 1990s and early 2000s. Sweet wormwood (*Artemisia annua*) extracts had historically been used for the treatment of malaria in China. The active compound of these extracts, called Artemisinin, was first isolated in 1979 (Figure 1.4) [White, Hien, and Nosten 2015]. The road to adoption of artemisinin derivatives as therapeutic agents was slow and convoluted but they have since become a mainstay of malaria control, with global adoption in the early 2000s [White, Hien, and Nosten 2015]. In order to achieve better pharmacological properties, including bioavailability, semi-synthetic derivatives of artemisinin are used as drugs [Paddon and Keasling 2014]. These include: artemether, dihydroartemisinin and artesunate, the latter being injectable and used for severe malaria (Figure 1.4). Initially these were used as monotherapies, but the short half-life of artemisinin derivatives and concerns over resistance, as well as the observation of significant levels of recrudescence (discussed below), prompted the development and implementation of artemisinin combination therapies (ACTs). These combine an artemisinin derivative with a companion drug that has a longer half-life to ensure adequate longevity of parasite killing to prevent the outgrowth of resistant parasites. The choice of partner drug for use with artemisinins in different settings primarily depends on tolerability, cost and pre-existing drug resistance [Davis et al. 2005]. Artemisinins are currently used in combination with lumefantrine, amodiaquine, piperaquine, mefloquine, sulfadoxine-pyrimethamine and pyronaridine [Tilley et al. 2016].

Artemisinin derivatives undergo intracellular reductive scission of the endoperoxide bridge by Fe^{2+} -haem [Blasco, Leroy, and Fidock 2017]. In their activated state these drugs are lethal to parasites, presumably through alkylation of biomolecules including haem, proteins and lipids, causing oxidative stress and cell damage [Tilley et al. 2016]. The high potency of artemisinins may therefore be due to the abundance of Fe^{2+} -haem in blood stage malaria parasites which results from breakdown of haemoglobin in the parasite's food vacuole. Artemisinins are highly active against trophozoite forms, in which haemoglobin catabolism reaches its peak. Unlike other antimalarials they are also active against ring forms [Xie et al. 2016]. This early activity likely results from the start of haemoglobin catabolism a few hours after erythrocyte invasion. When used against sensitive parasites

the artemisinin derivatives can achieve 10,000-fold reductions in parasite biomass every 48 h, providing exceptionally rapid clearance rates [Dondorp et al. 2009]. Unfortunately, resistance is emerging to ACTs, with parasites becoming resistant to both the artemisinin derivatives and partner drugs [Blasco, Leroy, and Fidock 2017]. Delays in parasite clearance times were first reported in 2008 in Cambodia [Anderson et al. 2017] and are now observed across Southeast Asia [Thu et al. 2017]. The same region, which was the crucible for evolution of resistance to both chloroquine and sulfadoxine-pyrimethamine, appears to be giving rise to resistant parasite strains once again [Blasco, Leroy, and Fidock 2017]. Resistance to ACTs has been linked to mutations in the Kelch-like K13 gene, which have now swept across Southeast Asia [Ariey et al. 2014; Ghorbal et al. 2014]. The failure of chloroquine was estimated to have resulted in a six-fold increase in mortality of children with malaria [Trape 2001] and mathematical modelling has suggested that in the face of widespread resistance to ACTs an additional 100,000 people will die of malaria every year [White, Pukrittayakamee, et al. 2014]. The failure of ACTs will therefore represent a public health disaster. As yet, ACTs are not failing outright and the intrinsic sensitivity of parasites remains, although some believe it is merely a matter of when, rather than if, this happens. Even in the absence of outright failures to clear parasites, recrudescence, whereby parasites apparently clear and symptoms abate, only to return following cessation of treatment causes great problems for the treatment of malaria [Rea, Holder, and Tewari 2017]. Indeed Artemisinin monotherapies, despite their high potency have always been associated with high rates of recrudescence [Teuscher, Gatton, et al. 2010]. It has been found that increasing treatment regimen from <5 days to 5-10 days reduces recrudescence rates [Sy et al. 1993]. Importantly re-treatment with the same artemisinin derivative is as effective as the initial treatment suggesting that recrudescence arises through a mechanism other than development of resistance [Teuscher, Gatton, et al. 2010; Docherty et al. 2003; Noedl, Se, et al. 2008; Phyo et al. 2012]. It is not clear why such a potent drug is associated with such high rates of recrudescence and two hypotheses have been formed to explain this phenomenon. One hypothesis is that the recrudescence occurs because the very short half-life of artemisinins results in plasma drug concentrations not remaining above the minimum inhibitory concentration to kill all parasites. There is some *in vitro* and clinical evidence to support this. Exposure of parasites to artemisinin *in vitro* for only 3 h was insufficient to eradicate parasites, which could only be achieved with extended dosing regimen [Bwijo and Hassan Alinz 1997]. In a clinical trial in Thailand decreased rates of recrudescence inversely correlated with drug concentration and dosing frequency [Looareesuwan 1994]. An alternative mechanism has been proposed in which parasites are able to enter a dormant state of growth arrest similar to that seen for some bacteria [Odenholt, Lowdin, and Cars 1997]. There is now a significant body of work supporting the existence of dormant ring stage parasites that can resume normal growth and it has been shown that this occurs rapidly after exposure to dihydroartemisinin (DHA) [Codd et al. 2011; Grobler et al. 2014; Lacrue et al. 2011; Teuscher, Gatton, et al. 2010; Teuscher, Chen, et al. 2012]. Recently a study has shown that phosphorylation of eIF2 α (elongation initiation factor α) by PK4 (protein kinase 4) is associated with DHA treatment and is critical for DHA induced dormancy of rings by repressing general translation [Zhang et al. 2017]. In fact resistance to artemisinins has been proposed to involve an altered dormancy pro-

file [Cheng, Kyle, and Gatton 2012]. The emergence of parasites with resistance to ACTs that take longer to clear, compounds the issue of recrudescence as parasites are then not exposed to the minimum inhibitory concentration of drug and escape clearance by the artemisinin derivative. Hopefully a combination of extending the treatment regimens, administering triple combination therapies, rotating partner drugs (as well as drug classes more generally) and the development of new synthetic endoperoxides will maintain the useful therapeutic life of this invaluable class of antimalarials [Phillips et al. 2017; Tilley et al. 2016].

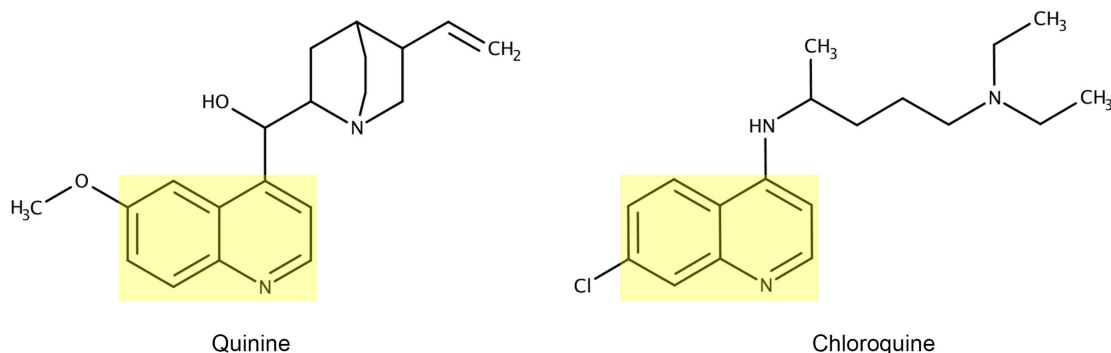
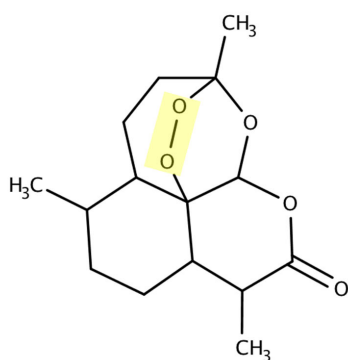
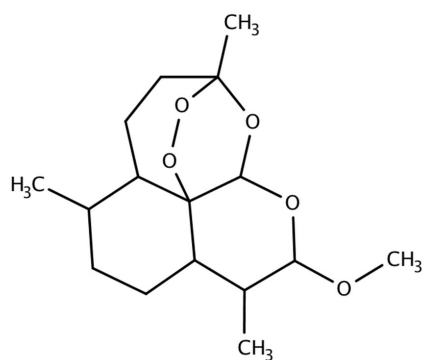


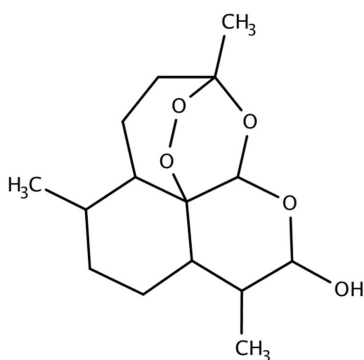
Figure 1.3: **Chemical structure of quinine and the related chloroquine.** The widely used drugs quinine and chloroquine are both based around the heterocyclic aromatic quinoline scaffold (highlighted yellow).



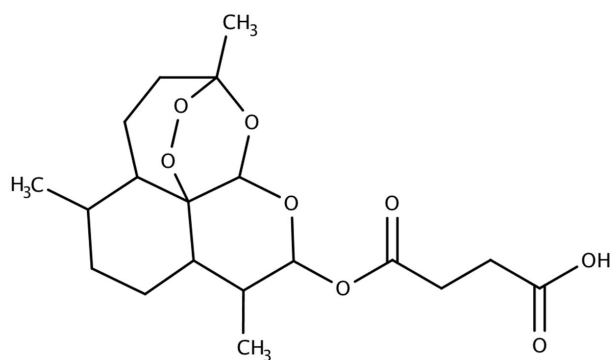
Artemisinin



Artemether



Dihydroartemesinin



Artesunate

Figure 1.4: **Chemical structure of artemisinin and some of its derivatives.** This shows the originally discovered artemisinin and some of the common derivatives of this molecule that are widely administered in artemisinin combination therapies for the treatment of malaria. The endoperoxide bridge responsible for the mechanism of action of artemisinin derivatives is shown shaded yellow. This becomes cleaved by Fe^{2+} haem iron leading to free radical generation which kills the parasite.

1.9 The future of antimalarial chemotherapy

Given the emergence of resistance to ACTs there is great interest in developing novel drugs, particularly ones with novel modes of action in order to circumvent development of cross resistance. Medicines for Malaria Venture (MMV) lists five target candidate profiles for new antimalarials: molecules that clear blood stage parasites, molecules that clear hypnozoites, molecules that clear hepatic stage schizonts, molecules that block transmission by killing gametocytes and molecules that block transmission by targeting the vector. Drugs are sought that are active against all species and strains of *Plasmodium* and active enough, or have a long enough half life that a single dose is required to ensure compliance. As valuable as artemisinins are in combatting malaria, they do not kill mature gametocytes which are responsible for transmission [Tilley et al. 2016] or the dormant hypnozoite form of *P. vivax* that gives rise to relapsing infection and is an important reservoir for transmission [Markus 2010; White, Pukrittayakamee, et al. 2014]. With eradication back on the global health agenda, there is great interest that the pipeline of drug development produces drugs that also act on gametocytes and hypnozoites in order to break the transmission cycle [Phillips et al. 2017]. While the screening of great numbers of compounds for their effects on hypnozoites remains some way off, researchers are taking steps to develop assays including high-content screens that begin to make this a reality [Wells, Burrows, and Baird 2010; Chattopadhyay et al. 2010].

High throughput phenotypic screening involves the direct assessment of compounds on proliferation or survival of parasite cells. In an early screening study, researchers assembled a large number of drugs already approved by the FDA or undergoing phase II clinical trials and examined their effects on *Plasmodium* [Chong et al. 2006]. The rationale behind such an approach is that, having undergone approval, these drugs have already been proven safe and can be ‘clinically repositioned’ and rapidly rolled out if they possess antimalarial properties. Alternatively, medicinal chemistry studies can be undertaken on a relatively well understood compound scaffold. In this study the non-sedating antihistamine astemizole was identified as a promising new inhibitor of *Plasmodium*. Such approaches have since been used to screen many small molecules. Such an approach has identified a number of lead compounds that might be developed as drugs [L. H. Miller, Ackerman, et al. 2013]. This includes a spiroindolone NITD609 that was produced following lead compound identification from a library of 12,000 natural products and synthetic compounds [Rottmann et al. 2010]. NITD609 kills erythrocytic stages (including gametocytes) with nano molar potency, and acts through a novel mechanism, believed to be inhibition of PfATP4, a transporter in the parasite plasma membrane which regulates Na⁺ and H⁺ homeostasis [Phillips et al. 2017].

The two other most commonly-used approaches to drug development are target based screening, whereby library screening is performed to assess inhibition of a validated target, and rational design. The former has identified inhibitors of *P. falciparum* dihydroorotate dehydrogenase, cytochrome bc1 and NDH2 [L. H. Miller, Ackerman, et al. 2013]. As yet, the rational approach has only really been applied to the modification of existing classes of drugs to generate new variants and overcome specific pharmacological challenges, such as generating derivatives of artemisinin with improved bioavailability. In

the drive to develop new drugs that has taken place in the last 10-15 years, phenotypic screening has been the most successful at uncovering new compound hits. Phenotypic screening will continue to be important but as more becomes known about the molecular and cellular biochemistry of the parasite, especially with the advent of new technologies including CRISPR Cas9 genome editing and Cre recombinase technologies to discover and validate drug targets, the more these targeted approaches will bear fruit.

1.10 The ability to propagate various malaria parasites enables the study of this disease

A prerequisite to conducting studies of a pathogen is the ability to propagate it continuously either *in vitro* or *in vivo*. The asexual blood stage cycle of *P. falciparum* has been propagated *in vitro* in human erythrocytes since Trager and Jensen developed the candle jar method [Trager and Jensen 1976]. It has been possible to continuously culture *P. knowlesi* in cynomolgus macaque erythrocytes but limited access to these precludes this for most laboratories. Recently however Moon and colleagues adapted *P. knowlesi* to propagate efficiently in human erythrocytes which will facilitate work on this species [Moon, J. Hall, et al. 2013; Moon, Sharaf, et al. 2016]. *P. knowlesi* is significantly more amenable to genetic manipulation in addition to having merozoites which are larger and less labile than those of *P. falciparum*, which will aid microscopic examination and invasion studies [Grüring et al. 2014]. Currently it is impossible to propagate any of the other human infecting species *in vitro* which hampers the investigation of their biology. *In vitro* culture allows the investigation of parasite molecular genetics, biochemistry and cellular biology, but obviously does not allow investigations of host related phenomena such as pathology, immunological responses and population dynamics during infection. This requires the use of animal models of malaria infection. The human infecting parasite species have a limited host range however [Siu and Ploss 2015]. Apart from humans, *P. falciparum* and *P. vivax* can probably only infect large apes such as gorillas and chimpanzees. Studies in chimpanzees led to the demonstration that hypnozoites result from *P. vivax* infection [Krotoski et al. 1982] and led to the identification of drug-resistance loci [Walliker et al. 1983]. The fact that *P. knowlesi* infects macaque monkeys as well as humans has also meant significant numbers of studies have been performed using macaques [Pasini et al. 2016]. Additionally, some studies have been conducted into *P. cynomolgi*, a parasite of Aotus monkeys as it is closely related to *P. vivax* and *P. knowlesi* [Joyner et al. 2016]. The use of monkeys and apes for research is prohibitive however. They are exceedingly expensive and difficult to work with, there are ethical concerns and their outbred genetics limits reproducibility of studies [Siu and Ploss 2015]. As such, most malaria research involving animal models makes use of rodent infecting species including *P. berghei*, *P. yoelii* and *P. chabaudi*. Given that part of the *Plasmodium* lifecycle takes place in the mosquito vector, researchers are often interested in studies of these stages as well. While mosquito infection can be established in the laboratory with many if not all species of *Plasmodium* without the need for animals, it is generally much more amenable with the rodent malaria species, not to mention considerably safer than working with mosquitos infected with human malaria parasites. As such the malaria models of infection are typically used for

studies into aspects of parasite biology during the mosquito stage. Additionally *P. berghei* is much more amenable to genetic modification than *P. falciparum*. Despite how important these models have been and will continue to be for understanding malaria, there are difficulties in translating our discoveries in these models to human disease given the physiological and genetic differences between both the hosts and the parasites. For example, the rodent parasite genomes lack orthologs for ~730 genes in *P. falciparum* [N. Hall et al. 2005]. To help overcome this, a humanised mouse model has been developed in which the organs and tissues have been engineered to be human-like to allow studies on *P. falciparum* *in vivo* [Siu and Ploss 2015]. This model will require further refinement but holds great promise. For the foreseeable future though, we will continue to require a combination of studies using the various existing malaria models, making use of the strengths and advantages of each to piece together an improved understanding of the disease and pathogen.

1.11 The use of conditional systems of genetic modification in *P. falciparum*

In order to study the cellular biology, biochemistry and molecular genetics of an organism it is often necessary, or at least desirable, to perturb the genetics of the organism in some way. Transfection of a malaria parasite was first reported over twenty years ago in *P. gallinaceum*, a parasite of birds [Goonewardene et al. 1993]. This was followed by similar work in *P. falciparum* [Wu et al. 1995] and *P. berghei* [Dijk, Waters, and Janse 1995]. Many other species including *P. knowlesi*, *P. cynomolgi*, *P. yoelii* and *P. chabaudi* are now routinely transfected with exogenous DNA for overexpression, gene disruption, reporter gene expression and epitope tagging studies [Koning-Ward, Gilson, and Crabb 2015]. Transfection is always performed in the asexual blood stage cycle due to its accessibility. The first gene disruption in *P. falciparum* was achieved twenty years ago [Crabb et al. 1997]. Gene knockouts have become routine, often using the reliable human DHFR gene as a drug marker to select for parasites in which the desired gene manipulation has occurred [Fidock and Wellems 1997] and the PlasmoGem project is an example of how gene knockouts can be used in *P. berghei* in a powerful and systematic way [Schwach et al. 2015]. Gene knockouts pose a problem for the investigation of genes essential to the asexual blood stage cycle however, as perturbations to a gene that affect an essential function will abrogate the viability of parasites; if they cannot be isolated, they cannot be studied. In contrast, this is not a problem for the study of genes that are essential only during the sexual blood stages or the insect stages; parasites can be maintained and loss of viability will only occur following transition to these other stages. As such, a conditional system of mutagenesis has been sought for use in *P. falciparum* and other *Plasmodium* spp for some time. Various systems have been developed including the FLP/FRT system in *P. berghei* [Lacroix et al. 2011]. What follows is an overview of the different conditional systems of genetic manipulation that have been investigated or implemented in *P. falciparum*.

1.11.1 Transcriptional control: The use of tetracycline-induced systems of transcriptional control in *P. falciparum*

One of the most widely used inducible systems for investigating the molecular genetics of eukaryotes is the Tet-repressor (TetR) based system of transcriptional activation. This system makes use of the tetracycline-dependent binding of TetR protein to 19 nucleotide DNA sequences called Tet operators (TetO). In Gram-negative bacteria in which this system exists naturally, the TetR, true to its name, functions as a repressor of gene transcription when bound to TetO repeats upstream of the promoter region by preventing binding of transcription factors. Tetracycline binds to TetR, preventing it from binding to the TetO repeats and thus activating transcription of the downstream operon. Gossen and Bujard made use of the DNA binding function of TetR to transform it from a repressor to an activator of gene expression in mammalian systems by fusing it to a C-terminal domain of the herpes simplex virus transcriptional activator VP16 [Gossen and Bujard 1996]. This fusion of TetR and VP16 is referred to as the TetR transactivator (tTA). TetO sequences were placed upstream of a minimal cytomegalovirus (CMV) promoter to drive expression. The addition of tetracycline (or tetracycline analogues such as anhydrotetracycline) prevents binding of the tTA to the promoter region and significantly reduces gene expression. Systems that function in this manner are referred to as Tet-off systems as the effect of tetracycline is to turn expression off. There also exist Tet-on systems whereby the tTA consists of a mutant TetR which has a reversed Tet-sensitive response. In this case tetracycline binding to the tTA is necessary for binding to the TetO and activation of transcription. Therefore addition of tetracycline turns transcription on.

Unfortunately, this widely used TetR/VP16 fusion was found not activate minimal promoters in the related Apicomplexan *Toxoplasma gondii* [Meissner, Brecht, et al. 2001] and it is assumed *P. falciparum* as well. In light of this, these authors attempted translational repression with TetR alone, as naturally occurs in Gram negative bacteria, but this was of limited effectiveness. The generation of an artificial transactivator (TATi) was therefore developed in *T. gondii* [Meissner 2002] which was used in *P. falciparum* with success [Meissner, Krejany, et al. 2005; Gilson, O'Donnell, et al. 2008; O'Neill et al. 2011]. The Apicomplexan ApiAP2 transcription factors have been used to generate a TetR fused transactivator [Pino et al. 2012]. A significant drawback to the use of these systems however is the requirement for transactivator-responsive minimal promoters that mimic the transcriptional profile of the native promoter, especially important given that *Plasmodium* genes are generally regulated at the transcriptional level and highly temporally regulated. Problematically, every minimal promoter tested in *T. gondii* has been found to have residual activity in the absence of bound transactivator [Jiménez-Ruiz et al. 2014]. The system is impractical for control of endogenous genes, as modifying the promoter regions of *P. falciparum* is technically difficult. Double crossover homologous recombination is inefficient [Duraisingh, Triglia, and Cowman 2002] and the targeting of integration constructs into the highly AT rich intergenic sequences by single crossover homologous recombination also problematic. These sequences are difficult to amplify by PCR and propagate in *E. coli* and targeting integration specifically into one of the many highly AT rich regions is difficult [Epp, Raskolnikov, and Deitsch 2008]. While control of episomal reporter gene expression has been achieved in *P. falciparum* using this method, control of endogenous

genes has not owing to the difficulty in manipulating promoters [Prommana et al. 2013].

1.11.2 Methods of transcript destabilisation

RNA interference (RNAi) does not function in *Plasmodium*

RNAi is a system of post-transcriptional genetic regulation in many eukaryotes that functions by destroying RNA transcripts. It is hypothesised that the ancestral role of RNAi was to maintain genome stability by controlling mobile DNA elements but it can also function as a system of intrinsic immunity against viruses, destroying their genetic material and as a means of regulating gene expression of an organism's own genes. RNAi technology has been utilised extensively in a wide range of organisms as a means of genetic manipulation and there is also much interest in its therapeutic potential [Wilson and Doudna 2013]. RNAi technology has been implemented in various protozoan parasites, most notably in the African Trypanosome *Trypanosoma brucei*, where it has enabled the development of a system for genome wide screening [Alsford et al. 2012]. Double stranded RNA molecules (dsRNAs) added exogenously or expressed within the cell are cleaved into short interfering RNA molecules (siRNAs) which target the RNA induced silencing complex (RISC) to the homologous mRNA transcripts. The endonuclease component called Argonaut (Ago) then mediates their degradation. Obviously there has been much interest in establishing the use of RNAi in *Plasmodium*. Various attempts have been made by multiple laboratories over the years, initially resulting in conflicting and ambiguous results. For example, one study which cultured *P. falciparum* in the presence of dsRNAs targeting falcipain-1 concluded this gene was essential to parasite viability [Malhotra et al. 2002]. This finding was at odds with another study which demonstrated through standard gene disruption by targeted homologous recombination that falcipain-1 is not essential during the asexual blood stage cycle [Sijwali, Kato, et al. 2004]. A systematic experimental and comparative genomics study by Baum and colleagues finally came to the conclusion that *Plasmodium* lacks the enzymes necessary for RNAi and found no experimental evidence of RNAi based gene knockdown [Baum et al. 2009].

The glmS ribozyme system has been used successfully in *Plasmodium*

Ribozymes are RNA molecules capable of acting as enzymes, including with the capacity to self-cleave through endonuclease activity. The use of ribozymes with ligand-dependent self-cleaving activity has allowed conditional regulation of gene expression. The first use of this system was reported by Yen and colleagues who made use of the hammerhead Sm1 ribozyme of *Schistosoma mansoni* in a mammalian system [Yen et al. 2004]. This was engineered for increased activity in order to destabilise the mRNA of a reporter gene when the Sm1 sequence was fused up-stream of the ORF. The nucleoside analogue toyocamycin was found to abrogate ribozyme self-cleavage, stabilising mRNA transcripts and so up-regulating reporter gene expression. Agop-Nersesian and colleagues subsequently attempted to use this same ribozyme to achieve down-regulation of a reporter gene in *T. gondii* and *P. falciparum* [Agop-Nersesian et al. 2008]. Some regulation was achieved in *T. gondii*, but unfortunately toyocamycin was highly toxic in this system. This toxicity was

dependent on adenosine kinase (AK) and could be abrogated by knockout of AK but this also abolished the transcript stabilising effect of toyocamycin. *Plasmodium* was found not to possess AK, consistent with the fact that toyocamycin is not toxic to *Plasmodium*, but this also meant that the drug failed to inhibit ribozyme activity. In an improvement of this system, Promanna and colleagues made use of the glmS ribozyme from Gram-positive bacteria [Lee and Oh 2015], inserting the sequence downstream of a reporter gene. The self-cleavage activity of this ribozyme is activated upon addition of glucosamine which results in reduction of gene expression. This system has been used successfully for gene knockdown in *Plasmodium* [Mchugh et al. 2015].

1.11.3 Inhibition of translation: The TetR-aptamer system successfully regulates gene translation.

A TetR-aptamer system of translational control has been demonstrated to successfully regulate expression in *Plasmodium* [Goldfless, Belmont, et al. 2012; Goldfless, Wagner, and Niles 2014]. In this system TetR-binding RNA 'aptamers' are placed within the 5' UTR of a gene of interest. The binding of TetR to the aptamer region disrupts translation of mRNA, but the addition of anhydrotetracycline prevents this binding and allows translation to occur. This system has two significant advantages over both the tetracycline-sensitive transactivator and DD systems (see below): it requires no prior knowledge of transcriptional regulation, as the gene of interest remains under control of its native regulatory sequences save for the addition of the aptamer sequence; and it does not require tagging of the protein product. Furthermore, this system has the greatest potential to be tuneable.

1.11.4 Protein destabilisation: Use of destabilisation domains for knock-down of protein in *P. falciparum*.

In 2003, it was found that a mutant version of the rapamycin-binding FRB (fragment of rapamycin binding) domain of mTOR (mechanistic target of rapamycin) led to degradation of a fused protein in human fibroblasts, but that it was stabilised in a rapamycin-dependent manner [Stankunas et al. 2003]. This serendipitous discovery led to an elegant forward genetic study with the aim to create a 'single ligand, single binding domain' system for rapid, reversible and tuneable regulation of gene expression. This study identified a mutant form of another rapamycin binding protein, FKBP12, that is inherently unstable, leading to degradation of fused proteins. This destabilisation occurs in the absence of, but not in the presence of the rapamycin analogue shield-1. This mutant FKBP12 domain is therefore referred to as the destabilisation domain, or 'DD' [Banaszynski et al. 2006]. Successful use of this DD system in *Plasmodium* was established by Armstrong and Goldberg [Armstrong and Goldberg 2007] and it has subsequently been made use of in numerous studies on *P. falciparum* [Russo et al. 2009; Dvorin et al. 2010; Ganter et al. 2017; Blomqvist et al. 2017; Absalon, Robbins, and Dvorin 2016; Muralidharan et al. 2012]. Unfortunately, there are significant drawbacks to this system which prevent its universal use. Most significantly, the requirement to fuse a protein of interest to the DD precludes its use if doing so alters the function or localisation of the protein adversely. Indeed, in many

cases it is simply lethal, and therefore impossible, to fuse the DD to a protein. Furthermore, even where this is possible, the degradation relies on targeting the fusion protein to the proteasome and therefore this system is typically only applicable to cytosolic proteins [Azevedo et al. 2012]. Proteins trafficked to secretory organelles, membranes or secreted beyond the parasite (i.e. into the PV, the erythrocyte cytosol, erythrocyte membrane, or organelles such as Maurer's clefts) will likely be inaccessible to the proteasome. As such the DD system is simply not applicable to many proteins of interest as evidenced by the multiple failures to achieve knockdown using this system not just in *Plasmodium* but *T. gondii* as well [Hallée and Richard 2015; Jiménez-Ruiz et al. 2014]. Furthermore, given that this system acts to down-regulate transcript levels it achieves knockdown rather than true knockout. While this is clearly satisfactory (and may be useful) in some situations it is well documented that, with enzymes even significant knockdown can be insufficient to ablate function to a point where a phenotype can be observed [Méthot et al. 2007; Methot et al. 2008]. Furthermore shield-1 is somewhat toxic to *Plasmodium* at the concentrations required for protein stabilisation which can complicate the interpretation of results. In an attempt to overcome the shortcomings of shield-1 another destabilisation tag has been employed.

In order to overcome the issues of cost and toxicity of shield-1, another destabilisation tag has been used in *Plasmodium falciparum*. It is based on the *E. coli* dihydrofolate reductase (DHFR) enzyme and is termed the DHFR destabilisation domain (DDD) and was initially developed by Iwamoto and colleagues [Iwamoto et al. 2010]. This tag is stabilised by inexpensive folate analogues such as trimethoprim and Muralidharan and colleagues used this tag to achieve conditional destabilisation of both a fluorescent reporter protein and the *RPN6* gene in *P. falciparum*. A separate study by Beck and colleagues set out to use the DDD to achieve knockdown of HSP101, a component of the PTEX complex [Beck et al. 2014]. Disruption of HSP101 function was achieved following removal of trimethoprim, resulting in a loss of protein export, but not through any knockdown of HSP101 protein levels. This is likely due to the inability for the PVM resident HSP101 to access the proteasome in the parasite cytosol for degradation. The authors ascribe the loss of function observed in this study to interference of the DDD tag with chaperone function [Muralidharan et al. 2012; Beck et al. 2014].

The auxin inducible degron system is another system of protein destabilisation that has been implemented in both *P. berghei* [Philip et al. 2015] and *P. falciparum* [Kreidenweiss, Hopkins, and Mordmüller 2013]. This system, which is naturally present in plants, achieves ubiquitination and subsequent proteasome mediated degradation of proteins fused to an auxin inducible degron (AID). Auxin binds to the AID and acts as a 'molecular glue', stabilising interaction of the AID containing protein and a Skp1, Cullin1, F box protein ubiquitin ligase (SCF) which results in ubiquitination of the AID containing protein by the SCF. The ubiquitinated AID containing protein is then degraded by the proteasome. The impetus for the implementation of this system in *P. berghei* was that the afore mentioned DD systems rely on constant maintenance of small molecules, shield-1 or trimethoprim, making them often unsuitable for *in vivo* applications, especially in the non-erythrocytic stages of the parasite's lifecycle [Philip et al. 2015]. Again however, the reliance of this system on proteasome mediated degradation for gene knockdown means it will likely only

be applicable for the study of proteins present in the cytosol.

1.11.5 Manipulation of the gene: The dimerisable Cre recombinase system is a highly rapid and efficient system for conditional gene modification in *P. falciparum*.

Cre recombinase is an enzyme of bacteriophage P1 that mediates excision of DNA between specific sequences called *loxP* sites using a topoisomerase I-like mechanism [Sternberg and Hamilton 1981]. Cre recombinase was so named because a specific fragment of the bacteriophage genome containing the gene was found to Cause REcombination, and *loxP* sites because they were the LOcation of crossing (Xing), P1 [Sternberg and Hamilton 1981]. Initial attempts were made at adapting the Cre recombinase system to *Plasmodium* by but regulation of its activity by the Tet-off system proved ineffective [O'Neill et al. 2011]. Andenmatten and colleagues then made use of a rapamycin-induced dimerisable form of Cre recombinase in *T. gondii*. The use of this system was pioneered by Jullien and colleagues [Jullien 2003; Jullien et al. 2007]. At the time Cre recombinase was increasingly widely used to engineer the genomes of mammals, with regulation of its activity generally controlled using Tet-sensitive expression or hormonal control. A problem with these systems is that leaky activity of Cre recombinase results in premature excision of DNA. In the DiCre approach of Jullien and colleagues, the two major helical Cre recombinase domains were separated into two fragments, each fused to a different rapamycin binding domain, FKBP12 or FRB. These fragments displayed a very low level of activity. Induced dimerisation of the two components of Cre recombinase through addition of rapamycin rapidly reconstituted Cre recombinase activity, providing a means of inducing Cre recombinase activity at will.

The DiCre system was adapted for use in *P. falciparum* by Collins and colleagues [C. R. Collins, S. Das, et al. 2013], being initially used to inducibly remove the 3'UTR of the *SERA5* gene in anticipation that this would achieve destabilisation of transcripts and silencing of expression. This did not succeed owing to the presence of cryptic 3' polyadenylation sequences remaining following Cre-mediated DNA excision, but it clearly demonstrated the utility of Cre recombinase technology in *P. falciparum*. Furthermore, the work produced the 1G5DC clone of *P. falciparum* which constitutively expresses the two dimerisable fragments of Cre recombinase. This clone has subsequently been widely used in number of studies [C. R. Collins, Hackett, et al. 2017; S. Das et al. 2015; Jones et al. 2016] including the study presented in this thesis. This same technology has also been implemented in *P. falciparum* independently [Yap et al. 2014].

1.11.6 The use of plaque assays in studies on infectious organisms including *Plasmodium* spp

Much research on *P. falciparum* focuses on the identification and characterisation of potential drug targets and therefore requires an assessment of parasite growth in response to gene disruption or modification. As discussed, the asexual blood stage cycle acts to amplify the parasite population exponentially, rather like a viral lytic cycle. For many viruses, this lytic cycle has long been exploited in *in vitro* assays to determine the con-

centration of infectious viral particles in a sample by determining the number of areas of host cell destruction following their infection by suitably titrated aliquots of virus. First described for animal viruses in 1953 [Dulbecco and Vogt 1953], the assay protocol usually involves limiting diffusion of the released viral particles through the use of semi-solid media in order to achieve discrete, highly localised regions of host cell monolayer destruction called plaques. The remaining host cell monolayers are then stained to visualise plaques. Because of their simplicity and broad applicability, plaque assays are amongst the most valuable and widely-used tools in viral research, allowing facile quantification of the effects of environmental conditions, drugs, antibodies and genetic manipulation on viral replication, as well as simplifying isolation of viral clones. Plaque assays have also been developed for other intracellular pathogens, including several viruses and bacterial species [Edouard and Raoult 2016] as well as the Apicomplexan *T. gondii* which readily infects most nucleated mammalian cells and thus can be cultured in adherent fibroblast monolayers [Chaparas and Schlesinger 1959]. In contrast, blood stages of *Plasmodium* species replicate exclusively in erythrocytes (or reticulocytes), which are not normally adherent. Plaque assays developed for *Plasmodium* have therefore used monolayers of erythrocytes adhered to the base of tissue culture wells using concanavalin A [Bruce et al. 1990; Inselburg 1983], Cell-Tak [Williams 1999], or α -Rhesus D antibodies plus protein L [Smith et al. 2000], with plaque formation being visualised using either Giemsa staining of fixed monolayers or immunofluorescence. Such assays were key to the success of elegant pioneering experiments demonstrating the phenomenon in which all the merozoite offspring of a single infected erythrocyte are committed to either continuation of the asexual life cycle or transformation into either male or female gametocytes [Bruce et al. 1990; Inselburg 1983; Smith et al. 2000]. However, due to the single-cell thick nature of the adherent erythrocyte monolayers produced by these methods and the need for fixation and staining to visualise the plaques, the assays are unsuitable for routine quantification of malaria parasite growth rates. A simple plaque assay for use with *Plasmodium* species would be a valuable tool for research into blood stage malaria research.

1.12 Proteases are present throughout the tree of life.

Proteases are enzymes that mediate the hydrolysis of peptide bonds and conduct a wide variety of regulatory and effector roles across the whole breadth of the tree of life. These include diverse roles in biological processes such as protein homeostasis, trafficking, cell signalling, catabolism and cell death. Their roles may involve relatively promiscuous cleavage to degrade proteins to individual amino acids for the digestion of proteins, or specific cleavage of just one molecule at one site during proteolytic activation 'cascades'. Throughout nature, 'cascades' of proteolytic activation are a commonly used means of regulating enzymatic activity and amplifying signals. Protease cascades consist of a protease cleaving and converting a zymogen protease to an active form, which then itself goes on to activate another zymogen protease and so on. Particularly well understood examples of proteolytic cascades include those involved in caspase mediated apoptosis [Martin and Green 1995], blood coagulation [Davie and Ratnoff 1964; Macfarlane 1964], the matrix metalloproteinase cascade [Lochter et al. 1997] and the complement activation

cascade [Lynskey et al. 2017]. Proteases are attractive targets for rational drug design due to their well understood catalytic mechanisms and active site structures [Deu 2017; Amour et al. 2004]. There is good precedent for the development of inhibitors targeting proteases with drugs recently brought to market including inhibitors of HIV protease and hepatitis C virus protease. Proteases are categorised into seven broad classes based on their mechanism of action, referred to as: cysteine, serine, aspartyl, threonine, metallo, glutamic and asparagine proteases. This is determined by the residue, or co-ordinating ion that functions as the active site nucleophile when hydrolysing peptide bonds. Members of each class other than the glutamic and asparagine classes have been identified in *Plasmodium*, including confirmation of protease activity of multiple members [Deu 2017].

1.12.1 Proteases play roles in a variety of pathways essential to parasite survival

Proteases have been found to play roles in various biochemical pathways that are essential for malaria parasite viability, but the roles of many remain uncharacterised. Multiple proteases are known to be involved in haemoglobin degradation within the parasite food vacuole which provides amino acids for protein synthesis. In *P. falciparum* these include four aspartyl proteases (plasmepsins I-IV), three papain-like cysteine protease (falcipains 2, 2' and 3), the metalloprotease falcilysin and a cysteine exopeptidase, DPAP1 (dipeptidyl aminopeptidase-1) [Wang et al. 2011]. As mediators of such a crucial biochemical pathway there has been great interest in studying these proteases and developing inhibitors as potential chemotherapeutic agents. Unfortunately, significant redundancy was found to exist amongst these proteases with every falcipain having been knocked out individually [Sijwali, Koo, et al. 2006], and all of plasmepsins I-IV being knocked out simultaneously [Alfredo Bonilla et al. 2007] with only limited impact on parasite viability. As such, members of this haemoglobin degradation pathway are unlikely to be good drug targets after all.

Protein trafficking is a ubiquitous feature of eukaryotic cells that requires proteases. Most soluble proteins that are secreted are expressed with an N-terminal hydrophobic signal sequence that inserts into the endoplasmic reticulum (ER) membrane co-translationally. This is then cleaved from the rest of the protein by the signal peptidase complex. In *P. falciparum*, two components of this complex (SP18 and SP21) have been identified and reported to possess protease activity [Sharma et al. 2005; Tuteja, Pradhan, and Sharma 2008]. Inhibition of prokaryotic signal peptidase activity results in cell death due to accumulation of proteins intended for secretion and as such have been considered potential novel antibiotic targets for bacteria [Paetzel, Dalbey, and Strynadka 2000] raising the possibility that inhibitors of *Plasmodium* homologs might inhibit parasite growth. In addition, proteases are required to maintain protein homeostasis in the ER. Proteins can become misfolded and require degradation by the proteasome in a process termed ER-associated degradation (ERAD). One such component of the pathway in *P. falciparum*, signal peptide peptidase (SPP), was identified bioinformatically by homology with mammalian components of the system [Harbut et al. 2012]. The number of components in the ERAD pathway in *Plasmodium* and other protozoan pathogens is highly reduced compared to mammals and so this pathway may represent a vulnerable aspect of the parasite's response to cel-

lular stress. In the same study, inhibitors of SPP were found to exhibit low nano molar potency against blood and liver stage parasites with low toxicity in mammalian cells, highlighting the potential for the development of drugs to target SPP.

In order to survive inside an erythrocyte the parasite must modify the erythrocyte membrane and cytosol in order to import nutrients, adhere to the vascular endothelium and evade the immune system. In order to achieve this, approximately 10% of the parasite's proteome is exported beyond the PV [Schulze et al. 2015]. Export of proteins across the PVM is achieved via a still poorly-characterised complex termed the PTEX complex (*Plasmodium* translocon for exported proteins) [Gilson, Chisholm, et al. 2017]. Most exported proteins possess a PEXEL (protein export element) motif downstream of the secretory signal peptide. As part of the trafficking of PEXEL motif containing proteins the PEXEL motif is cleaved by *P. falciparum* plasmepsin V (an aspartyl protease) in the ER and upon the protein's arrival in the PV the exposed N-terminus is recognised by the PTEX complex (*Plasmodium* translocon of exported proteins)[Boddey et al. 2010; Russo et al. 2009]. Plasmepsin V is therefore an attractive drug target as abolition of its function is likely to interfere with export of proteins on a global level and consequently block a range of essential biological processes including metabolite import, haemoglobin internalisation, Maurer's cleft formation and modification of the erythrocyte cytoskeleton and erythrocyte surface.

1.13 Egress

1.13.1 Egress is fundamental to the lifecycle of intracellular pathogens.

Egress generally is the process by which an intracellular pathogen is released from the host cell in which it resides. All intracellular pathogens including viruses, bacteria and protist parasites must undergo some form of egress. Egress occurs at multiple points in the *Plasmodium* lifecycle: at the end of the liver stage infection, at the end of each intra-erythrocytic cycle during the asexual blood stage, of the gametocyte upon ingestion by the mosquito and of sporozoites from the oocyst in the mosquito midgut basal lamina. During the asexual blood stage cycle the newly formed merozoites must escape the confines of the depleted erythrocyte to invade fresh cells and propagate their population. *Plasmodium* egress is cytolytic and the direct destruction of erythrocytes, as well as the systemic damage caused by the ensuing inflammatory response, is responsible for all the clinical symptoms of malaria (discussed in Section 1.4) [Yahata et al. 2012]. Being the cause of pathology, and crucial to the continuation of infection, blood stage egress is an important aspect of parasite biology and a greater understanding is key to fully comprehending the pathology of this disease.

Over the years some controversy ensued as to the order of membrane rupture with an early study suggesting that the erythrocyte membrane ruptures before the PVM [Salmon, Oksman, and Goldberg 2001]. Over time it has become more apparent that this is incorrect, with a number of studies contributing to the growing body of evidence that the PVM ruptures before the host cell membrane not only in blood stage egress [Wickham, Culvenor, and Cowman 2003; Glushakova, Humphrey, et al. 2010], but also in gameto-

cyte egress [Sologub et al. 2011; Deligianni et al. 2013; Wirth, Glushakova, et al. 2014] and liver stage egress [Burda, Roelli, et al. 2015; Burda, Caldelari, and Heussler 2017; Schmidt-Christensen et al. 2008; Putrianti et al. 2010; Graewe et al. 2011].

1.14 Gametocyte egress, but not asexual blood stage egress, has been shown to be perforin dependent

Many pathogens use pore-forming proteins to achieve entry into host cells, release from vacuoles into the host cell cytoplasm or egress from their host cells [Rosado et al. 2008; Roiko and Carruthers 2009]. In *Toxoplasma* the perforin like protein TgPLP1 which contains a membrane attack complex/perforin (MACPF) domain has been shown to be involved in egress of tachyzoites from host cells [Kafsack et al. 2007]. While parasites deficient in this protein eventually egress, apparently through mechanical means, they show a delay in egress with PVM and host cell plasma membrane remaining intact. This suggests TgPLP1 plays a role in PVM rupture and potentially that of the host cell membrane as well. There also exists another *T. gondii* MACPF gene, *TgPLP2*, but no function is yet known for this protein [Wade and Tweten 2015].

This finding prompted interest in the five MACPF domain-containing *Plasmodium* perforin like proteins (PPLPs) that are encoded in the *P. falciparum* genome (PPLP1-5) [Kaiser et al. 2004]. PPLP1 and/or PPLP2 have been suggested to play a role in asexual blood stage egress of *P. falciparum* with both shown to be expressed during this stage and PPLP1 localised to the micronemes, with secretion into the PV observed [Garg et al. 2013]. Arguing against this, however, studies in *P. berghei* found that PPLP1 and PPLP2 are dispensable during the asexual blood stage cycle with PPLP1 important only for sporozoite cell traversal [Ishino, Chinzei, and Yuda 2005; Amino, Giovannini, et al. 2008] and PPLP2 for rupture of the erythrocyte membrane surrounding the gametocyte [Deligianni et al. 2013; Wirth, Glushakova, et al. 2014]. Furthermore, in a very recent study disruption of the *P. falciparum* PPLP1 gene was found to have no phenotypic consequences in asexual blood stages [Yang et al. 2017]. PPLPs 3-5 are all expressed in the ookinete stage [N. Hall et al. 2005; Raibaud et al. 2006]. PPLPs 3 and 5 have been shown to have functions in ookinete traversal of the mosquito midgut in studies on mouse malaria species *P. berghei* and *P. yoelii* [Kadota et al. 2004; Ecker, Pinto, et al. 2007; Ecker, Bushell, et al. 2008] while the same role has been demonstrated for PPLP4 in *P. falciparum* [Wirth, Bennink, et al. 2015]. The existing picture is therefore quite complex, with studies performed across different species and during different lifecycle stages. Further genetic studies of *in vitro* *P. falciparum* will be required to unambiguously determine whether any of the PPLPs play a role in egress of asexual blood stages and/or whether there is functional redundancy amongst these proteins.

1.14.1 Blood stage egress is a kinase and protease-regulated process

Plasmodium blood stage egress (as with egress in other stages of the lifecycle) is an active, rapid and highly regulated process taking place over the course of only a few minutes at the end of each erythrocytic cycle and occurs synchronously, i.e. all daughter mero-

zoites are released simultaneously [Blackman 2008; Blackman and Carruthers 2013]. The necessity of this regulation and synchronicity is evident from the fact that *Plasmodium* undergoes replication by schizogony as outlined in Section 1.3. Premature egress would be catastrophic as only very mature parasites undergo cytokinesis and merozoites might not have even formed let alone be properly prepared for successful invasion of a fresh erythrocyte. As evidence of this, a recent study demonstrated that the conditional disruption of SERA5 results in egress occurring prematurely. These mutants egressed inefficiently, with merozoites still bound by residual erythrocyte membrane, in turn resulting in reduced invasion rates [C. R. Collins, Hackett, et al. 2017]. This suggests that the kinetics of egress must be tightly controlled, even within the final ~ 10 min of the blood stage cycle, to achieve efficient merozoite release and successful invasion. This is in contrast to *T. gondii* tachyzoites which replicate by repeated rounds of binary fission (endodyogeny) which results in the continuous formation of viable progeny. Induced egress of *T. gondii* by treatment with the calcium ionophore A23187 results in productive invasion of fresh host cells. What is quite clear is that following activation of the parasite's cyclic-GMP (cGMP) dependent protein kinase (PKG) a number of highly choreographed cellular events take place during the asexual blood stage cycle: the contents of exonemes and micronemes (specialised secretory organelles of merozoites) are discharged into the PV [C. R. Collins, S. Das, et al. 2013], the PV swells to fill the erythrocyte as the erythrocyte 'rounds up', transforming almost instantaneously from an irregular shape to a roughly spherical one [Glushakova, Humphrey, et al. 2010]; the PVM ruptures to release merozoites into the residual erythrocyte cytoplasm [Wickham, Culvenor, and Cowman 2003; Glushakova, Yin, et al. 2005]; the erythrocyte membrane becomes 'porated' [Glushakova, Humphrey, et al. 2010; Glushakova, Busse, et al. 2017] as the cytoskeleton apparently collapses [Hale et al. 2017]; and finally, the erythrocyte membrane ruptures in an elastic fashion to achieve merozoite release [Abkarian et al. 1994] (Figure 1.8). It is also apparent that at some point just prior to or during this pathway the PVM becomes porated, allowing mixing of the PV contents with those of the residual erythrocyte cytosol [Hale et al. 2017]. Apart from the overarching regulation of these events by PKG however, none of these relatively well-described cellular events have been ascribed to specific molecular effectors during egress of the asexual blood stage parasite. What follows is a review of what is known about the cellular and biochemical processes involved in *Plasmodium* blood stage egress, with reference to egress during other lifecycle stages and of other species where relevant.

PKG is a key regulator of *Plasmodium* egress

PKG is a key signalling hub involved in regulating multiple transitional phases in the parasite lifecycle in both the mammalian host and mosquito vector [Alam et al. 2015; Baker et al. 2017]. PKG is stimulated through an increase in the second messenger cGMP and its activation is essential for triggering the egress of merozoites during both the liver stage [Falae et al. 2010] and blood stage [M. C. Taylor, Kaur, et al. 2008; H. M. Taylor et al. 2010; C. R. Collins, S. Das, et al. 2013]. PKG is also involved in regulation of gametogenesis [McRobert et al. 2008; M. C. Taylor, Kaur, et al. 2008], ookinete gliding in the mosquito midgut [Moon, C. J. Taylor, et al. 2009] and sporozoite invasion of the hepato-

cyte [Govindasamy et al. 2016]. PKG is therefore likely essential across the entire parasite lifecycle, making it an attractive drug target as inhibition may have transmission blocking effects in addition to clearing parasites from the patient's blood stream [Baker et al. 2017]. During the asexual blood stage, pharmacological blockade of PKG with the selective and reversible inhibitors compound 1 (C1) and compound 2 (C2) [Gurnett et al. 2002; Donald et al. 2006] abrogates discharge of micronemes and exonemes with a concomitant block in egress [C. R. Collins, S. Das, et al. 2013]. Wash off of C1/C2 allows merozoite release to occur within ~10 minutes, indicating the role of PKG in triggering egress. As such, these reversible inhibitors of PKG have become invaluable tools in the study of egress. This will be discussed specifically in Section 1.16. As PKG is stimulated through elevation of cGMP levels it follows that steady state levels of cGMP must be carefully controlled in the parasite and this is likely achieved through interplay between cGMP-degrading phosphodiesterases (PDEs) and guanylyl cyclases (GC) that up-regulate cGMP levels (Figure 1.5). Collins and colleagues demonstrated that treatment of mature blood stage schizonts with zaprinast, an inhibitor of PDEs, stimulates egress of wild-type parasites, an effect that was blocked by the PKG inhibitors C1/C2 [C. R. Collins, S. Das, et al. 2013]. When the same experiment was performed on parasites expressing a PKG mutant possessing an enlarged active site gatekeeper residue that abolishes C1/C2 sensitivity, zaprinast was found to induce egress in the presence of C1/C2. These results are consistent with activation of PKG occurring through a tightly regulated rise in cGMP levels, triggering egress (Figure 1.5).

The natural egress signal is unknown but it is likely to be endogenous to the parasite and probably functions by either down-regulating PDE activity, or up-regulating the activity of GCs that enhance cGMP levels. The *P. falciparum* genome contains four putative PDEs, (α - δ) and two GCs (α and β). PfPDEs α , γ and δ are dispensable during the asexual blood stage cycle while PfPDE β is refractory to disruption [Wentzinger et al. 2008; M. C. Taylor, Kaur, et al. 2008; Moon, C. J. Taylor, et al. 2009]. GC α is refractory to disruption in both *P. falciparum* [M. C. Taylor and Kelly 2010] and *P. berghei*, [Moon, C. J. Taylor, et al. 2009] suggesting it is required during the asexual blood stage cycle, whereas GC β has been disrupted with no phenotype in blood stages [Hirai et al. 2006; Moon, C. J. Taylor, et al. 2009]. As such PDE β and GC α , both of which are apparently essential during the asexual blood stage cycle and involved in cGMP homeostasis are good candidates for the target of the natural egress signal. The PDE inhibitor zaprinast which appears to mimic this egress signal presumably inhibits PDE β . An understanding of how PKG triggers egress during the asexual blood stage, or indeed any of the other lifecycle events mentioned above, remains obscure but it appears linked to calcium signalling.

Cytosolic calcium signalling has been implicated in the regulation of egress. *Toxoplasma* tachyzoite egress involves mobilisation of calcium from internal stores [Carruthers and L. D. Sibley 1999; Lovett and L. D. Sibley 2003] and a spike in calcium levels has been observed just prior to egress of *P. falciparum* blood stage schizonts [Agarwal et al. 2013]. Furthermore, a calcium chelator was found to inhibit egress in *Plasmodium*, as in *Toxoplasma*. The inhibitory effects of this chelator were overcome by zaprinast-induced activation of PKG, suggesting that either PKG acts to increase calcium levels, or it functions downstream or independently of calcium [C. R. Collins, S. Das, et al. 2013].

This calcium flux in *P. falciparum* prior to egress was also investigated by Glushakova and colleagues. They demonstrated that calcium flux and egress were unaffected by the presence of the non-cell-permeable calcium chelator EDTA in the culture medium, suggesting that the intracellular calcium flux is derived from internal stores, rather than from external sources. Corroborating this idea, egress was enhanced by treatment with either a calcium ionophore (which allows calcium ions to cross lipid bilayers) or inhibitors of calcium pumps that sequester calcium in the ER. It was concluded by these authors that the ER is therefore the most likely source of the calcium signal [Glushakova, Lizunov, et al. 2013]. Consistent with this, a study of ookinete gliding (which requires PKG) found PKG-dependent phosphorylation of enzymes involved in metabolism of IP₃, a secondary messenger, which is responsible for liberating calcium ions from the ER. The enzymes included phospholipases which metabolise IP₃. This led these authors to suggest that one of the key roles of PKG is to regulate free cytosolic calcium levels through regulation of phospholipases which metabolise IP₃ [Brochet et al. 2014]. A recent phosphoproteomic study on asexual blood stages has identified numerous substrates that become phosphorylated in a PKG-dependent manner [Alam et al. 2015]. These data should begin to shed light on signalling pathways during blood stage egress.

The role(s) that calcium flux plays in egress remains unclear but at least one of its functions may be to activate the calcium-dependent protein kinase 5 (CDPK5) which has been shown to be essential for egress and so is a good candidate for the target of activation by the calcium flux [Dvorin et al. 2010]. The specific role of CDPK5 in egress is not known however. It is not required for discharge of exonemes or micronemes which are apparently central to egress/invasion as discussed below. How these signalling pathways intersect remains quite unclear. Given the calcium dependent nature of CDPK5, it presumably functions downstream of PKG activation in view of the role of PKG in mediating the calcium flux prior to egress. *Plasmodium* egress does not require motility [Frénal et al. 2017] so this cannot be the role of CDPK5 in achieving egress, although it is possible it has a role in activating the actinomyosin motor for invasion. It has been suggested that CDPK5 regulates the activity of factors involved in egress that might include proteases, pore forming proteins and lipases. These would presumably have to be discharged from secretory organelles though and given that exoneme and microneme discharge occur following PKG activation, independently of CDPK5 [C. R. Collins, S. Das, et al. 2013], it seems unlikely this is the case. It is possible that the functional diversity of these secretory organelles is greater than we currently appreciate and there may be subsets of exonemes and micronemes which do require CDPK5 activity for their secretion, parallel to those exonemes and micronemes, containing SUB1 and AMA1 respectively, that are secreted in the absence of CDPK5. Somehow though CDPK5 delivers an essential signal, separate to that of the SUB1/SERA5/SERA6 putative proteolytic pathway that is the focus of this PhD project and discussed below.

In summary, PKG is clearly essential for triggering egress and appears to be involved in invasion as well. Its role in invasion is less obvious however, as the labile nature of *P. falciparum* merozoites makes dissecting true secondary effects on invasion, away from general defects resulting from a primary effect on egress, difficult. PKG is clearly involved in secretion of exonemes and micronemes and is likely involved in mobilising a calcium

flux from internal stores (probably the ER) which may activate an additional parallel egress signal through CDPK5.

Asexual blood stage egress is a protease dependent process

For many years it has been known that egress is a protease dependent process [Blackman 2008; Blackman and Carruthers 2013]. Early studies showed that broad spectrum protease inhibitors targeting cysteine and serine type proteases interrupted the parasite lifecycle, trapping mature parasites in 'clusters' and blocking the infection of fresh erythrocytes [Lyon and Haynes 1986]. A global proteomic study found that over 180 *Plasmodium* proteins are cleaved during the 6 h leading to egress [Bowyer et al. 2011]. Host calpain-1 in the erythrocyte cytosol has been demonstrated to facilitate egress; merozoite release was found to be deficient in calpain-1 immuno-depleted erythrocytes [Chandramohanadas et al. 2009]. Calpain-1 is the most abundant cysteine protease in erythrocytes and calpains become activated by elevated levels of calcium and associate with membranes [Goll, Thompson, and Li 2003]. Whether a calcium flux in the erythrocyte does activate calpain-1, and how this is achieved is unknown. Zinc-dependent metalloproteases have also been implicated in egress/exflagellation of microgametes with inhibitors of this class of proteases preventing exflagellation [Sologub et al. 2011].

The parasite subtilisin like serine protease SUB1 has also been suggested to play a role in egress, conjectured to act by triggering a proteolytic pathway when discharged from exonemes into the PV following PKG activation [Yeoh et al. 2007]. *SUB1* is conserved across all *Plasmodium* species [Withers-Martinez, Strath, et al. 2014]. Studies in *P. berghei* have demonstrated that SUB1 is required for rupture of the PVM during liver stage infection [Tawk et al. 2013; Suarez et al. 2013]. During the asexual blood stage cycle, SUB1 is maximally expressed late in schizont development and was found to be refractory to non-conditional gene disruption, suggesting an essential role [Yeoh et al. 2007]. Pharmacological blockade of *P. falciparum* SUB1 (PfSUB1) activity was found to block egress [Yeoh et al. 2007]. PfSUB1 is expressed as an 82 kDa zymogen form possessing a prodomain [Blackman, Fujioka, et al. 1998]. Following cleavage of the signal sequence within the endoplasmic reticulum, PfSUB1 undergoes autocatalytic processing which separates the 28 kDa N-terminal prodomain from the remaining C-terminal segment (called p54) which contains the catalytic domain. The prodomain initially remains tightly associated with p54 [Blackman, Fujioka, et al. 1998; Sajid, Withers-Martinez, and Blackman 2000]. The p54 form in association with its prodomain is then trafficked to exonemes via the Golgi. At some point during this trafficking, the prodomain dissociates from the p54 form and a further autocatalytic processing step occurs resulting in a final 47 kDa product that accumulates in exonemes in highly mature schizonts [Sajid, Withers-Martinez, and Blackman 2000; Yeoh et al. 2007].

Upon its PKG-regulated release from exonemes into the PV, PfSUB1 is known to cleave an abundant merozoite surface protein called MSP1 [Koussis et al. 2009], as well as PfSERA5 and PfSERA6 [Yeoh et al. 2007; Ruecker et al. 2012] which are members of the multi-gene serine repeat antigen (SERA) family of putative papain like cysteine proteases (see Section 1.14.2). A diverse range of additional potential PfSUB1 substrates has also been identified which includes proteins in various compartments including the

PV, rhoptries and the erythrocyte [Silmon de Monerri et al. 2011].

Another protease, a dipeptidyl aminopeptidase (DPAP), called PfDPAP3, was previously implicated in playing a role during egress [Arastu-Kapur et al. 2008]. This study found that specific inhibition of PfDPAP3 blocked the PfSUB1-mediated processing of PfSERA5. The authors proposed a model in which PfDPAP3 directly or indirectly regulates the proteolytic maturation of PfSUB1. However recent work (as yet unpublished) has demonstrated this not to be the case, and that DPAP3 appears to be important for efficient RBC invasion instead (personal communication Christine Lehmann). Another *P. falciparum* DPAP, DPAP2 has been found to be expressed only in gametocyte stages [Tanaka2013]. A more recent study found that DPAP2 resides in secretory organelles called osmiophilic bodies and disruption of DPAP2 was found to decrease gamete egress [Suarez-Cortes2016].

1.14.2 Members of the serine repeat antigen (SERA) family are putative proteases implicated in playing roles during egress

The SERA family is a multi-gene family found in a tandem cluster in all *Plasmodium* species as well as the closely related genus *Theileria* (Figure 1.6) [Arisue et al. 2011] (Figure 1.6). The number of SERA genes varies between species, from just two in the avian parasite *P. gallinaceum* to 12 in *P. vivax* [Arisue et al. 2011]. *P. falciparum* possesses nine SERA genes with SERAs 1-8 on chromosome 2 and *SERA9* on chromosome 9. The SERA genes all possess a central papain-like cysteine protease domain and reside within the PV [Aoki et al. 2002; Delplace et al. 1987; Debrabant et al. 1992]. The SERAs are classified as 'Cys-type' or 'Ser-type' depending on the residue present in the active site nucleophile position of the papain domain. Canonical cysteine proteases possess a Cys residue in this position as it mediates protein hydrolysis by nucleophilic attack [Hodder et al. 2009; Rosenthal 2011; Deu, Verdoes, and Bogyo 2012]. It is therefore intriguing that some members of the SERA family have substituted this Cys with a Ser residue. This Cys to Ser substitution is expected to abolish protease activity in a cysteine protease scaffold. Corroborating this, no phenotypic consequences arose from substitution of the *PfSERA5* active site Ser with an Ala residue, which is expected to abolish protease activity of a cysteine protease as it cannot function as a nucleophile [Stallmach et al. 2015]. It is inferred from the fact that *Theileria* possesses one SERA gene of the Cys-type that this is the ancestral form of the SERA family [Arisue et al. 2011]. Furthermore, *P. gallinaceum* which represents an out-group to the mammalian-infecting species possesses only Cys-type SERAs. The mammal-infecting *Plasmodium* species possess Ser-type SERAs in varying numbers, leading to the hypothesis that a gene duplication event and Cys to Ser substitution took place in the common ancestor of all mammalian-infecting parasites, with subsequent gene duplications, deletions and mutations to pseudogenes leading to variable numbers of Ser-type genes present in the parasite species that infect mammals. All mammal infecting *Plasmodium* species possess three Cys-type SERA genes that show clear orthology across species. As such they can be classified into three groups [Arisue et al. 2011]. *PfSERA8* and orthologs form group I, *PfSERA7* and orthologs form group II and *PfSERA6* and orthologs form group III. The Ser-type genes do not form clear orthologous relationships across species but together form a distinct monophyletic group called

group IV. Interestingly the duplication of Ser-type genes has been particularly extensive in primate infecting *Plasmodium* species, the significance of which is unclear [Arisue et al. 2011]. The group I SERAs, including *PfSERA8*, stand apart in the SERA family as they are the only members whose expression is confined to the insect stages [Aly and Matuschewski 2005]; they also lack apparent SUB1 processing sites [Yeoh et al. 2007]. Work on *P. berghei* *SERA5* (also referred to as ECP1), the ortholog of *P. falciparum* *SERA8*, has shown it is expressed exclusively in mature oocysts and is necessary for sporozoite egress [Aly and Matuschewski 2005]. The same study also showed that oocysts lacking the *PbSERA5* gene cannot be permeabilised by saponin to allow IFA staining of circumsporozoite protein (CSP). The authors therefore suggested that in wild-type parasites, *PbSERA5* mediates proteolytic degradation of the oocyst wall. Work in *P. falciparum* and *P. berghei* has shown that the group II, group III and group IV SERAs localise to the PV in late trophozoite and schizont stages [S. K. Miller et al. 2002; Putrianti et al. 2010] and that they are SUB1 substrates [Yeoh et al. 2007; Ruecker et al. 2012]. In *P. falciparum*, gene deletion studies found that only *PfSERA5* and *PfSERA6* could not be disrupted in the asexual blood stage cycle through non-conditional approaches, suggesting that they have important roles [Ruecker et al. 2012; S. K. Miller et al. 2002; McCoubrie et al. 2007]. The cleavage of *PfSERA5* and *PfSERA6* following PKG mediated discharge of SUB1 has implicated them in playing roles in this process. Conditional disruption of *PfSERA5* has now demonstrated that it plays a role in regulating the kinetics of egress with merozoite release occurring prematurely (by ~3 min) and inefficiently; merozoites remain bound by residual erythrocyte membrane [C. R. Collins, Hackett, et al. 2017]. This results in a noticeable replication defect but *PfSERA5* is dispensable under standard *in vitro* conditions.

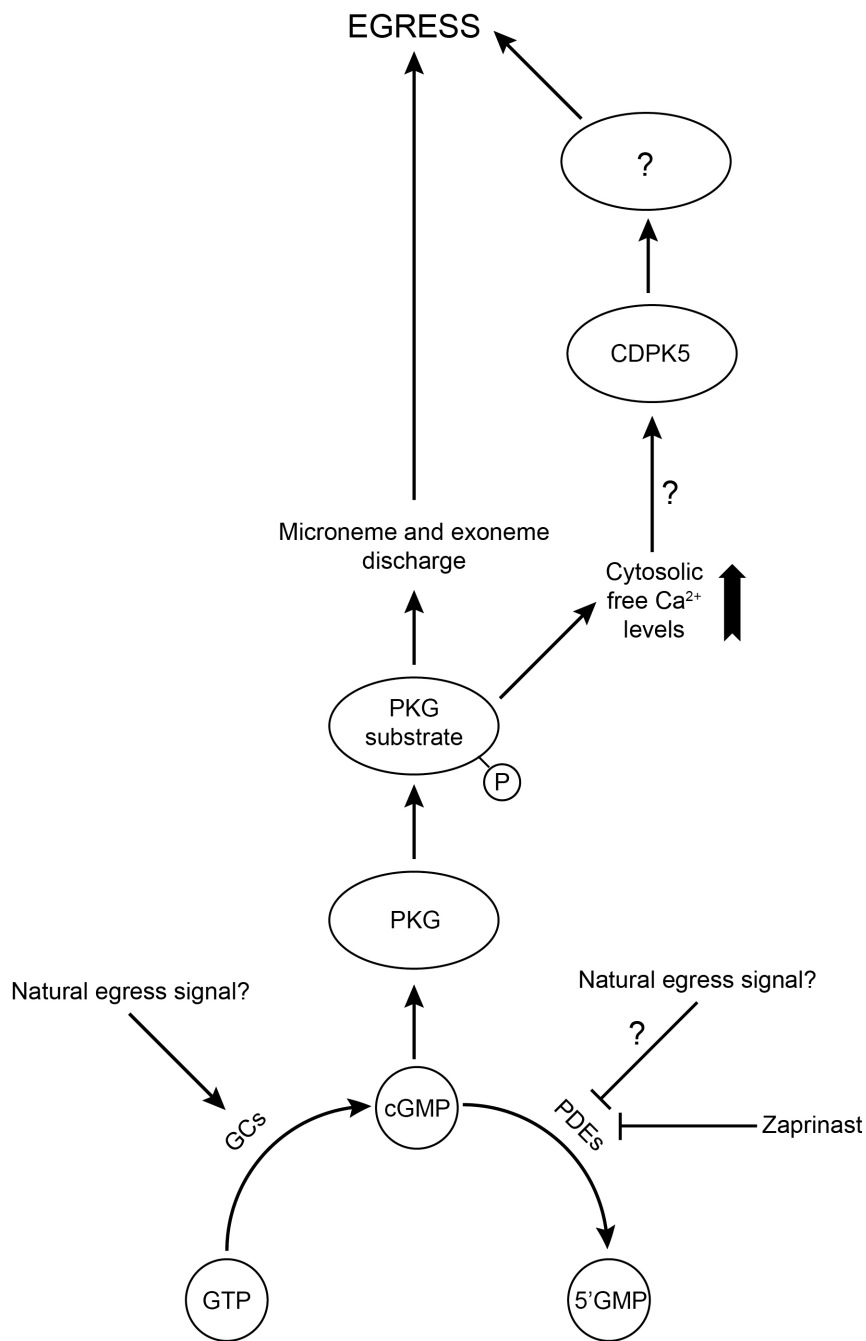


Figure 1.5: **A model of how PKG might signal for egress to occur.** Elevated levels of cGMP activate PKG which delivers signals required for the release of exonemes and micronemes. PKG is also believed to mediate a calcium flux in the cytosol which may be the mechanism by which CDPK5 (which is also required for egress) is activated. The endogenous egress signal which causes elevated levels of cGMP is unknown but is hypothesised to act by inhibiting the activity of one or more cGMP degrading PDEs, or upregulating the activity of GCs that metabolise cGMP.

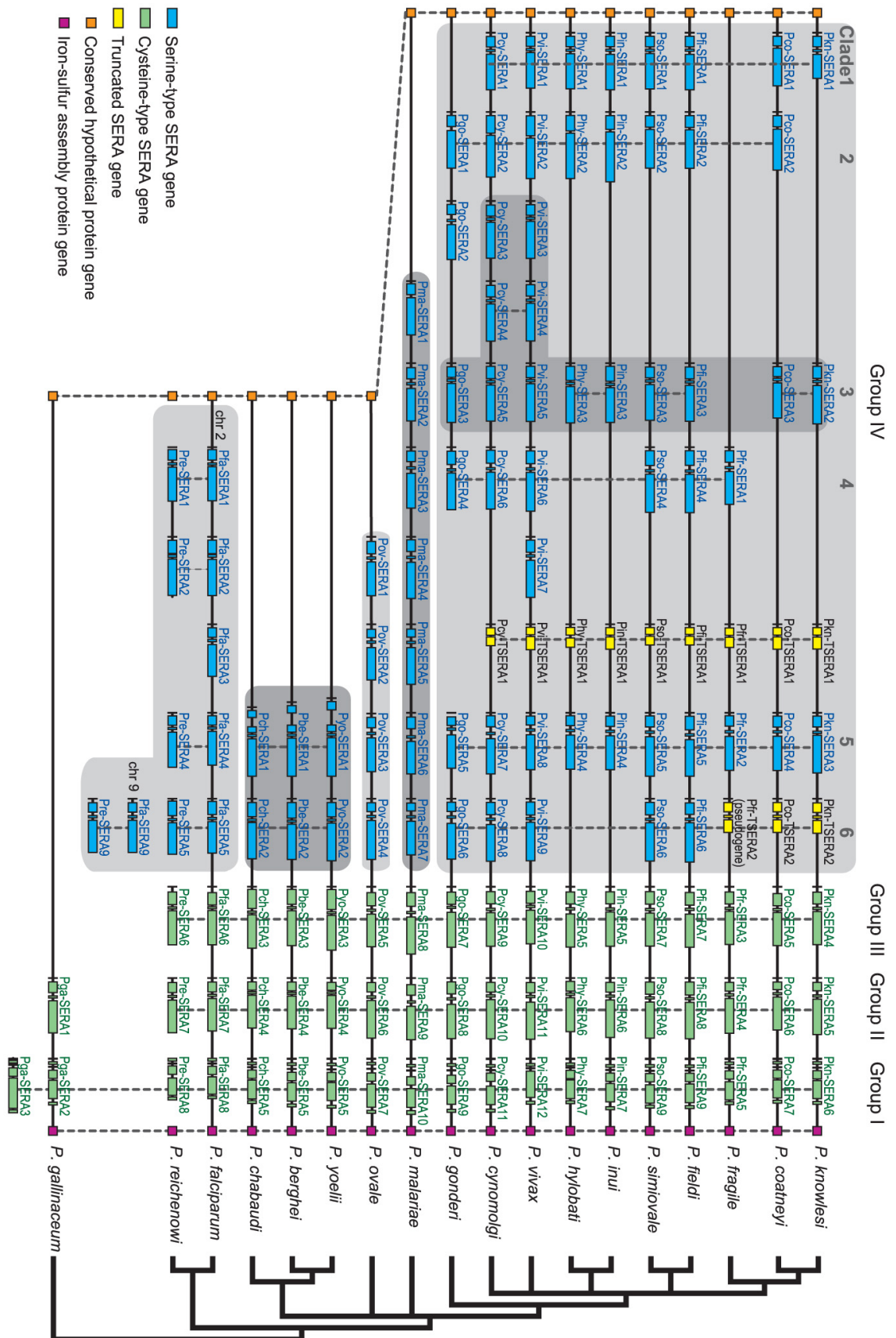


Figure 1.6: **Schematic of the SERA multi-gene family in various species of *Plasmodium*.** Clear homology and synteny exists across the group I, II and III Cys-type SERAs in all species examined other than *P. gallinaceum*. The group IV Ser-type SERAs exist in varying numbers in the different species. From [Arisue et al. 2011].

1.15 PfSERA6 may be an essential protease involved in egress

PfSERA6, with its active site Cys⁶⁴⁴, possesses the hallmark features of a cysteine protease (Figure 1.7). The failure to disrupt the *PfSERA6* gene by targeted homologous recombination [S. K. Miller et al. 2002] or to substitute the active site Cys⁶⁴⁴ with an Ala residue [Ruecker et al. 2012], suggest that *PfSERA6* plays an essential proteolytic role during the asexual blood stage cycle. Furthermore, studies of the *P. berghei* ortholog of *PfSERA6*, *PbSERA3*, showed that it appeared to possess an autocatalytic protease activity *in vitro*, [Ruecker et al. 2012]. The activity of *PbSERA3* was found be sensitive to E64, an epoxide based irreversible inhibitor of cysteine proteases and a compound known to inhibit merozoite release [Blackman 2008; Blackman and Carruthers 2013]. *PfSUB1* was shown to process *PfSERA6* either side of the papain domain just prior to erythrocyte rupture, suggesting that *PfSERA6* may be expressed as a precursor zymogen before conversion to an active enzyme within the final minutes before merozoite release [Ruecker et al. 2012]. As mentioned, various studies have found that E64 prevents parasite egress and so these findings raised the possibility that *PfSERA6* is the physiologically relevant target of E64 in inhibiting egress.

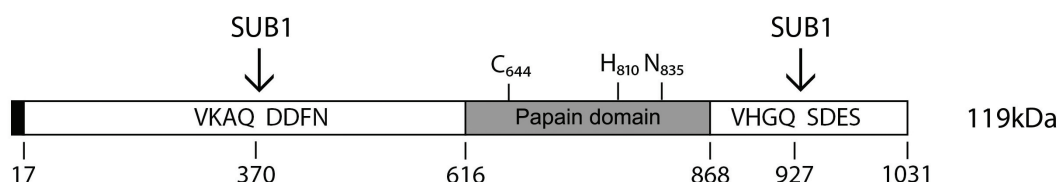


Figure 1.7: **Schematic representation of the primary structure of *PfSERA6*.** This figure shows a representation of the primary sequence of *PfSERA6*, showing the signal sequence (solid black), papain like domain (shaded grey), and the sites at which *SUB1* cleaves the protein either side of the papain domain. The amino acid sequence of the *SUB1* cleavage sites are shown, and the amino acid residue numbers of certain features are shown below, with protein size in kilo Daltons.

1.16 The reversible PKG inhibitors compound 1 and compound 2 and the irreversible cysteine protease inhibitor E64 have become invaluable tools for investigating blood stage egress

The study of egress has greatly benefited from the ability to selectively arrest two different stages in the pathway of egress. The reversible inhibition of PKG activity with C1/C2 has been particularly useful as it allows the preparation of highly synchronous cultures of mature schizonts that are stalled approximately 10-15 minutes before egress (Figure 1.8) [C. R. Collins, Hackett, et al. 2017]. Wash-off of C1/C2 reverses this inhibition of PKG and allows egress to proceed. This allows high proportions of a population to undergo egress within a very short space of time, greatly facilitating microscopic and biochemical studies of this process. E64, as discussed, is an irreversible inhibitor of cysteine proteases that prevents egress. The wash-off of C1/C2 in the presence of E64 arrests parasite egress at a point downstream of PKG activation when merozoites become readily discernible by DIC microscopic imaging which is the point at which PVM rupture has occurred [Glushakova, Humphrey, et al. 2010]. This later stage of arrest is also very useful as PKG activation has occurred, allowing the effects of PKG activation to be examined, which would be difficult or impossible if the egress pathway was allowed to proceed all the way to merozoite release. This approach ensures that parasites are only exposed to E64 when highly mature and less than 15 minutes away from merozoite release. This ensures that off-target effects of E64 are limited.

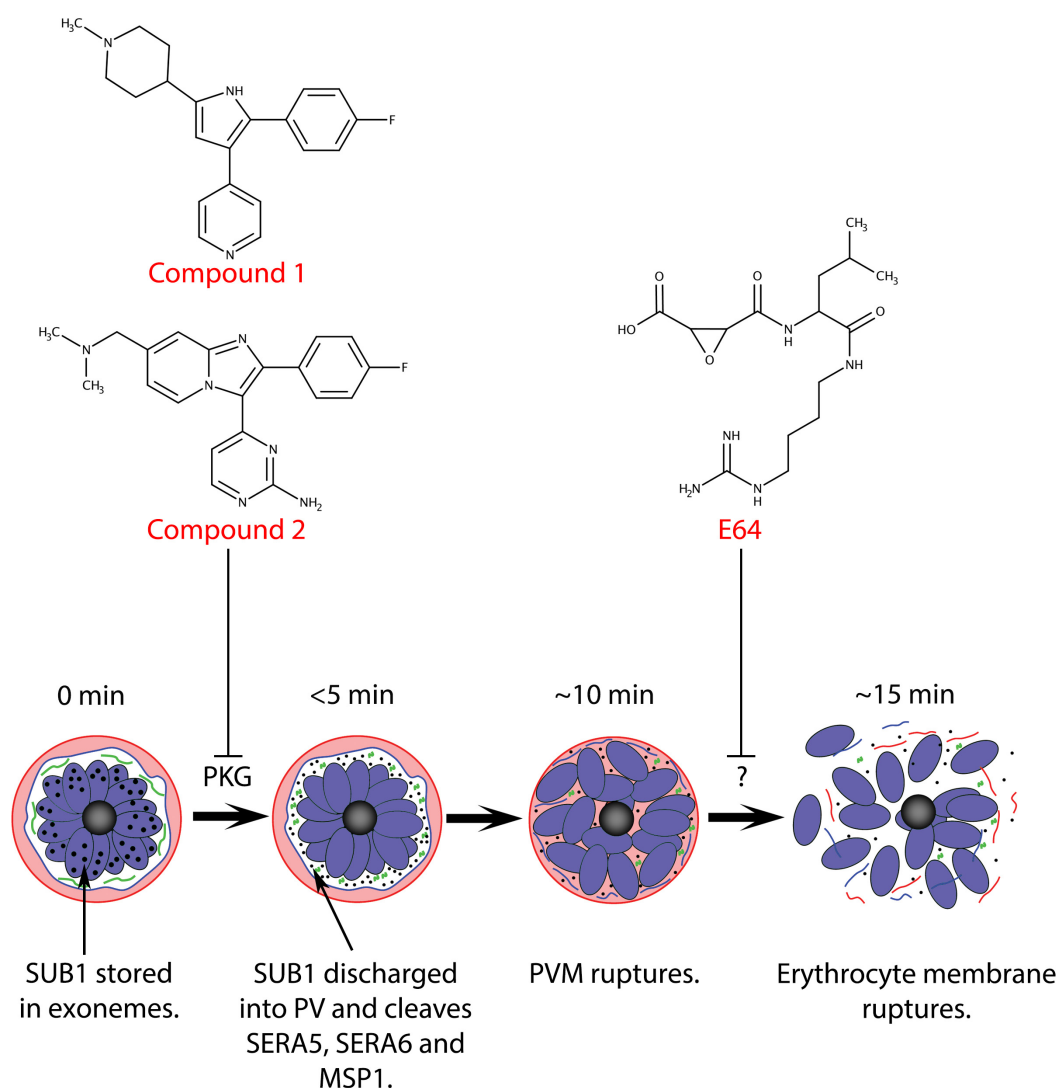


Figure 1.8: **Diagrammatic representation of the physical and cellular events leading to egress during the asexual blood stage.** PKG triggers egress. PKG and therefore egress can be reversibly inhibited with compound 1 or compound 2. Following PKG activation the contents of exonemes (which contain SUB1) are discharged into the PV. The contents of micronemes are also discharged which results in AMA1 relocation to the merozoite surface. In the PV SUB1 cleaves multiple substrates including MSP1, SERA5 and SERA6. The PVM then ruptures, followed by the erythrocyte membrane leading to merozoite release. At some point during this process the erythrocyte membrane becomes permeable [Glushakova, Humphrey, et al. 2010]. E64 can be used to inhibit egress at a point when the PVM has ruptured but erythrocyte membrane rupture has yet to occur. The target of this protease inhibitor relevant for achieving a block in egress remains unknown.

Chapter 2

Aims of this project

As discussed in the Introduction, the existing data at the outset of this project indicated that both SUB1 and SERA6 play roles in mediating merozoite release from the erythrocyte. The SUB1 processing of SERA6, a putative papain-like cysteine protease, resembled a classical proteolytic activation step converting a zymogen form to an active protease form. As such this project set out to address the following three outstanding questions regarding asexual blood stage egress in *P. falciparum*:

- Is SUB1 required for egress?
- Is SERA6 required for egress?
- Is SERA6 a protease and does its function require SUB1 processing?

To address these questions a molecular genetics approach has been used, making use of the DiCre system to conditionally silence the expression of *SUB1* and *SERA6*, independently, in order to examine the biochemistry and cell biology of these mutants.

Chapter 3

Methods

3.1 Culture and transfection of *P. falciparum* and *P. knowlesi*

3.1.1 Maintenance and synchronisation of parasite cultures

P. falciparum (wild type clone 3D7, the DiCre-expressing clone 1G5DC [C. R. Collins, S. Das, et al. 2013], MSP1:loxPint [S. Das et al. 2015; Jones et al. 2016] or clones and lines generated in this study, which were produced on the 1G5DC genetic background) were routinely cultured at 37°C in human erythrocytes at 1-4% haematocrit in RPMI 1640 (Life Technologies) supplemented with 2.3 gL⁻¹ sodium bicarbonate, 4 gL⁻¹ dextrose, 5.957 gL⁻¹ HEPES, 0.05 gL⁻¹ hypoxanthine, 0.5% (w/v) Albumax II, 0.025 gL⁻¹ gentamicin sulphate, and 0.292 gL⁻¹ L-glutamine (complete medium) in a 90% nitrogen, 5% carbon dioxide and 5% oxygen atmosphere as previously described [Trager and Jensen 1976; Yeoh et al. 2007]. Human blood was obtained from anonymised donors through the UK National Blood Transfusion service and was used within 2 weeks of receipt. No ethical approval is required for its use. Routine microscopic examination of parasite growth was performed by fixing air-dried thin blood films with 100% methanol before staining with 10% Giemsa stain (VWR international) in 6.7 mM phosphate buffer, pH 7.1. For routine microscopic determination of parasitaemia, at least 1,000 erythrocytes were examined. Exact haematocrit values were determined using a haemocytometer (Marienfeld; 0.1 mm depth and 0.0025 mm² area).

For synchronisation of parasite cultures, mature schizont stage parasites were isolated on cushions of 63% (v/v) Percoll (GE Healthcare Life Sciences) adjusted to isotonicity as described previously [Kramer, Chow Kan, and Siddiqui 1982]. Schizonts were then added to fresh suspensions of erythrocytes in complete medium and incubated at 37°C with shaking (~60 rpm) for 3-4 h to allow egress of merozoites and invasion of fresh erythrocytes. Remaining schizonts were then removed from the culture by isolation on percoll. Lysis of mature forms of the parasite that remained in the erythrocyte pellet was performed by suspension of the pellet in 5% (w/v) D-sorbitol [Lambros and Vanderberg 1979]. The synchronous ring stage parasites were then cultured as standard.

Plasmodium knowlesi A1.H1 clone parasites were cultured as *P. falciparum* but complete medium was supplemented with 10% (v/v) horse serum [Moon, J. Hall, et al. 2013; Moon, Sharaf, et al. 2016], and human erythrocytes used in cultures were pooled from three donors in case any one individual donor was Duffy negative which would prohibit *P.*

knowlesi invasion [L. H. Miller, Mason, et al. 1975].

3.1.2 Transfection of *P. falciparum*

For transfection of DNA constructs, Percoll-enriched mature synchronous schizonts were electroporated with 10 µg of sterile ethanol precipitated DNA per transfection using an Amaxa P3 primary cell 4D Nucleofector X Kit L (Lonza) (pulse code FP158). The DNA (resuspended in 10 µl) was mixed with 100 µl of P3 Primary cell solution from the Lonza kit. Then 20 µl of packed schizonts (previously washed after isolation on percoll in RPMI 1640 without albumax or supplementary glutamine) were resuspended in the DNA/P3 Primary Cell Solution mix, before transferring to a cuvette and being electroporated. After electroporation the contents of cuvettes were immediately added to 2 ml of complete medium at 15% haematocrit and shaken gently at 37°C for 1 hour to encourage invasion of single merozoites per erythrocyte, as for invasion during synchronisation. This was then made up to 10 ml of culture with fresh complete medium and incubated as standard (stationary overnight). Growth medium was replaced ~20 h post transfection with fresh complete medium containing drug. Once drug-resistant parasites appeared and displayed sustained growth (usually 2-3 weeks post-transfection, aliquots of culture were cryopreserved (see Section 3.1.5). In cases where episomal constructs were expected to integrate, cultures were subjected to repeated cycles of 3 weeks of culture without drug followed by culturing with drug until parasite growth recovered to sustained levels ('drug cycling'). This enriches for parasites in which the DNA construct has integrated into the genome. After each round of drug cycling the integration of the construct was assessed by diagnostic PCR to examine the presence of the native, unmodified chromosomal locus and the modified, integrant locus. When clear evidence of integration was detected parasites were cloned either by limiting dilution of cultures to ~0.1-0.3 parasites per well followed by dispensing into round-bottomed 96-well microplates and screening for parasite growth by examination of Giemsa stained thin films, or by serial dilution of culture and determination of clonal parasite growth by examination of plaques (see Section 3.1.4). Plates were incubated in sealed gassed incubator chambers.

3.1.3 Reagents

The antifolate drug WR99210 (from Jacobus Pharmaceuticals (New Jersey, USA)) was used at 2.5 nM. Blasticidin (used at 2 µg/ml) and rapamycin (used at 100 nM) were from Sigma. Compound 2 was kindly provided by Dr Simon Osborne (MRC Technology, Stevenage Bioscience Catalyst Campus, UK) and used at 1 µM.

3.1.4 Imaging and documentation of plaque formation

To allow plaque formation, synchronous parasite cultures at ring stage were diluted to the desired densities in complete medium with human erythrocytes at haematocrits between 0.5% and 1%, then dispensed into the central 60 wells of flat-bottomed 96 well microplates (Costar 3596, Corning NY, USA; 0.32 cm² growth area per well, diameter 6.38 mm) using 200 µl culture per well. Plates were incubated in sealed, gassed incubator chambers

(Billups-Rothenberg, CA, USA). To limit evaporation, sterile phosphate buffered saline was added to the outer wells of plates. Plaque formation was assessed routinely by microscopic examination using a Nikon TMS inverted microscope (4x objective, 10x eyepieces, 40x total magnification). When desired, plaque formation was documented without opening the plates using a Perfection V750 Pro scanner (Epson) in top-down transmission light mode, saving images as 4,800 dpi RGB TIFF files. Up to 3 microplates were imaged simultaneously with the scanner. Plaque clarity and visibility was generally enhanced by using the 'Split channels' function of the Fiji distribution of ImageJ [Schindelin et al. 2012] to split the RGB TIFF file into red, green and blue data channels, then using the green channel image for quantification of plaque number and dimensions, since this provided maximum contrast between plaques and the surrounding erythrocyte layer. Plaques were counted by visual examination of the images and plaque size quantified using the Magic Wand tool in Adobe Photoshop CS5 using a tolerance setting value of 32. This tool delineates the perimeter of selected plaques and calculates plaque area in pixels. No area value was recorded for apparent plaques that could not be delineated by the tool.

3.1.5 Cryopreservation of *P. falciparum* cultures

P. falciparum cultures were cryopreserved in liquid nitrogen. Parasite cultures of approximately 5% ring parasitaemia were centrifuged and the erythrocytes resuspended in 28% (v/v) glycerol, 3% (w/v) sorbitol, (w/v) 0.65% sodium chloride in distilled water with 1 ml added to each cryovial and then frozen. To thaw parasites, the cryovial was warmed to 37°C before the 1 ml of thawed culture was added to 5 ml of sterile thawing solution (3.5% (w/v) NaCl). Cells were pelleted at 1,100 rpm for 3 min. The pelleted cells were washed once more in the same volume of thawing solution and finally resuspended in 10 ml complete medium and cultured as standard. Selective drug medium, if applicable was added ~20 h later.

3.1.6 Rapamycin treatment of parasites

Synchronous cultures of ring stage parasites (~4-14 h old) were divided equally and treated with 100 nM rapamycin, or an equivalent volume of DMSO as a solvent-only control (referred to as 'mock-treatment'), for 4 h at 37°C. Cells were then pelleted and washed once in Albumax II-free RPMI before culturing as standard in complete medium.

3.1.7 Parasitaemia counts by flow-cytometry

Parasites were fixed in 4% (v/v) paraformaldehyde (Sigma), 0.02% (v/v) glutaraldehyde (Sigma) in PBS for 30 min at 37°C and then diluted five-fold in PBS. Samples were stored at 4°C until required. Cells were stained with Hoechst 33342 (Thermo Fisher Scientific) (1:10,000) in PBS for 30 min at 37°C. Parasitaemia was determined using a BD FACS Aria flow cytometer. Plots of forward scatter area and side scatter area were used in order to gate for human erythrocytes. Subsequently doublet discrimination was performed by gating with plots of forward scatter width and forward scatter height. Another round of doublet discrimination was performed by gating on plots of side scatter width and side

scatter height. Finally infected erythrocytes were gated for as erythrocytes positive for DNA stain by plotting forward scatter area against UV-A fluorescence using a 450/50 filter.

3.1.8 Extraction of genomic DNA from *P. falciparum*

Parasite enriched pellets were obtained by haemolysis of pelleted erythrocytes in at least 5 pellet volumes 0.15% (w/v) saponin (Sigma) in PBS followed by centrifugation at 13,000 rpm for 5 min in a microfuge. The haemoglobin rich supernatant was aspirated and discarded and pelleted parasite material stored at -20°C until required. DNA was extracted from pellets using a DNeasy Blood and Tissue Kit (Qiagen) as per manufacturer's instructions, or using standard phenol chloroform extraction.

3.1.9 Preparation of protein lysates for SDS PAGE and western blot

For the solubilisation of proteins in pelleted parasite material or culture supernatants the sample was dissolved in standard Laemmli buffer (4 x concentrate: 8% (w/v) SDS, 40% (v/v) glycerol, 250 mM Tris-HCl, 400mM DTT, 0.008% (w/v) bromophenol blue) and immediately heated at 95°C for 5 min to denature proteins and reduce disulphide bridges. Residual solid material was then pelleted by centrifugation at 13,000 rpm for 3 min in a microfuge. The supernatant containing solubilised proteins was then subjected to SDS PAGE fractionation.

3.1.10 Diagnostic polymerase chain reaction (PCR)

Diagnostic PCR was performed using Kappa2G HotStart ReadyMix (Kappa Biosystems) as per manufacturer's instructions. Primers were used at 500 nM. PCR was carried out in a thermocycler and products examined by agarose gel electrophoresis as standard. Oligonucleotide primers were obtained from Sigma.

3.2 Molecular cloning techniques

3.2.1 PCR for molecular cloning applications

'Colony PCR' was performed with GoTaq Green Master Mix (Promega). PCR reactions were inoculated with a small amount of bacteria from a single colony using a sterile pipette tip. Primers were used at 400 nM. PCR for amplification of sequences to be used for DNA transfection constructs was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) as per manufacturer's instructions. Primers were used at 500 nM. PCR was carried out in a thermocycler and products examined by agarose gel electrophoresis as standard. Oligonucleotide primers were obtained from Sigma. Purification of PCR products for molecular cloning applications was performed using a QIAquick PCR Purification Kit (Qiagen) as per manufacturer's instructions.

3.2.2 Restriction enzymes

All restriction enzymes used were obtained from New England Biolabs and used according to manufacturer's instructions.

3.2.3 DNA ligation

Ligation of DNA was performed using a Rapid DNA Ligation Kit (Roche) as per manufacturer's instructions.

3.2.4 Ligation independent cloning

For ligation independent cloning the InFusion HD Cloning Kit (Clontech) was used as per the manufacturer's instructions.

3.2.5 Plasmid DNA preps

Plasmid DNA purification from *E. coli* was performed using a QIAprep Spin Miniprep Kit (Qiagen) or a HiSpeed Plasmid Maxi Kit (Qiagen) as per manufacturer's instructions.

3.2.6 Extraction and purification of DNA from agarose gels

Extraction and purification of DNA from agarose gels after electrophoretic separation was performed using a QIAquick Gel Extraction Kit.

3.2.7 *E. coli* and transformation

DH5 α and One Shot OmniMAX 2 T1 Phage-Resistant *E. coli* cells were used for transformation with plasmids. *E. coli* was grown at 37°C in standard lysogeny broth.

3.2.8 Nucleotide sequencing

Sanger sequencing of DNA constructs to verify correct construction was performed by Beckman Coulter Genomics or Source Biosciences.

3.3 Generation of plasmids

SUB1HA3:loxP and SERA6:loxP parasite clones were generated by single crossover homologous recombination into the 1G5DC genome using integration plasmids called pHH1_SUB1HA3_loxP and pHH1_SERA6_loxP respectively. In both cases, correct transcriptional regulation of the modified gene was assured by placement of the 3' UTR of the *P. berghei* dihydrofolate reductase thymidylate synthase gene (*PbDT 3' UTR*) downstream of the floxed coding sequence [C. R. Collins, S. Das, et al. 2013]. To target the *SUB1* gene, a chimeric gene fragment with a native 5' portion and a recodonised 3' portion was constructed by first amplifying the 5' sequence from *P. falciparum* 3D7 gDNA using primers JT-S1endo-F and JT-S1CO-R [Table 3.1], whilst the recodonised region was

amplified from plasmid pFastBac-sPfSUB1wt [Withers-Martinez, Saldanha, et al. 2002] using primers JT-S1CO-F and JT-S1synth-R [Table 3.1]. The amplicons were then fused in frame by means of overlapping extension PCR by inclusion of both products in a fresh PCR reaction including primers JT-S1endo-F and JT-S1synth-R [Table 3.1], and the product cloned into pGEM-T (Promega). A segment of this fragment was then excised using HindIII and KpnI and replaced with a similarly-digested *loxP*-containing synthetic intron (*loxPint*) (Geneart) [S. Das et al. 2015; Jones et al. 2016]. This intermediate vector was digested with HpaI and Xho I to liberate the *SUB1* sequence which was ligated into the plasmid pHH1_SERA5_LoxP1 [C. R. Collins, S. Das, et al. 2013] digested with the same enzymes to generate plasmid pHH1_SUB1HA3_loxP (Figure 5.4). Integration of this construct into the 1G5DC *SUB1* locus by homologous recombination was detected by diagnostic PCR with primers JT111-1p and JT111-2p [Table 3.1], and the floxed or excised *SUB1* locus was detected by PCR using primers JT111-1p and JT111-3p [Table 3.1].

To generate pHH1_SERA6_loxP, a chimeric *SERA6* gene fragment was excised from plasmid MWS36 (a kind gift of Michael Shea) by digestion with HpaI and NcoI. The chimeric sequence was cloned into plasmid pHH1_SERA5_LoxP (Figure 5.5). This plasmid was digested with HindIII and then blunted before digesting with NcoI before being ligated to the excised *SERA6* fragment to produce pHH1_SERA6_loxP which contained a single *loxP* site downstream of the *SERA6* stop codon. The *SERA6* chimeric sequence in this plasmid consisted of 920 bp of native sequence starting within the first intron, fused to synthetic recodonised sequence stretching to the stop codon. Integration of this construct by homologous recombination into the 1G5DC genome (which already contains a single genomic *loxP* site upstream of the *SERA6* locus) was designed to introduce a second *loxP* site downstream of the *SERA6* stop codon floxing the entire *SERA6* open reading frame and promoter region. Correct integration was detected by diagnostic PCR using primers SERA6-5'UTRb and SERA6-37 (Table 3.1), whilst the floxed or excised *SERA6* locus was detected with primers S65'UTRb-2 and S6EndoEx2Rev (Table 3.1).

For generation of plasmid constructs designed for transgenic expression of *SERA6*, a chimeric coding sequence and native promoter region was excised from plasmid MWS28 (a kind gift of Michale Shea) and ligated into plasmid pDC-mCherry-MCS (a modification of pDC2-mCherry [C. R. Collins, Hackett, et al. 2017]) by digestion with Sall and NcoI, giving rise to pDC2-wtSERA6 (WT *SERA6*). The sequence comprised 979 bp of putative promoter sequence upstream of the native *SERA6* ATG start codon, as well as 477 bp of the 5' segment of the coding sequence (including the first intron) fused in frame to a synthetic recodonised *SERA6* cDNA (Geneart) encoding the remainder of the ORF. Site-directed mutagenesis and sub-cloning steps were then used as previously described [Ruecker et al. 2012] to generate identical constructs containing di-leucine substitutions of the P1 and P2 positions at the *SUB1* processing sites 1 and 2 in *SERA6*, as well as a Cys⁶⁴⁴Ala substitution of the active site nucleophile, giving rise to plasmids pDC2-SERA6_uncleavable (Uncleavable *SERA6*) and pDC2-SERA6_Alamut (Cys⁶⁴⁴Ala) respectively (Figure 5.15). The 979 bp of native promoter region of *SERA6* used to drive expression from these episomal constructs consisted of approximately almost the entire 1028 bp region between the *SERA6* start codon and the stop codon of the upstream *SERA5* gene.

For transgenic expression of WT *SUB1* the pDC-mCherry-MCS plasmid was modified

such that the blasticidin deaminase (BSD) drug selection cassette and mCherry reporter gene were expressed from a single promoter by the use of the ribosomal T2A skip peptide [J. H. Kim et al. 2011; Szymczak et al. 2004; Wagner et al. 2013]. To do this, the BSD cassette was excised from pDC2-mCherry_MCS with *Apal* and *SacI* and the backbone re-ligated. The mCherry ORF was then excised by digestion with *AvrII* and *XhoI* and replaced with a synthetic gBlock (IDT) comprising the mCherry and BSD ORFs separated by the T2A sequence. This resulted in construct pDC2-mCherryT2ABSD_MCS in which these ORFs remained under control of the constitutive *P. falciparum* calmodulin (*CAM*) promoter (which remained from the original mCherry expression cassette). This vector was then linearised with *SnaBI*. The *P. falciparum* *SUB1* promoter sequence (1000 bp immediately upstream of the *SUB1* start codon) was amplified from *P. falciparum* 3D7 gDNA using primers PfSUB1_prom_for_infu and PfSUB1_prom_rev_infu (Table 3.1). Primers PfSUB1_synth_for_infu and PfSUB1_synth_rev_infu were used to amplify the recodonised synthetic *SUB1* ORF from pFastBac-sPfSUB1wt [Withers-Martinez, Saldanha, et al. 2002] and primers PbDT3UTR-for_infu and PbDT3UTR-for_infu were used to amplify the PbDT 3'UTR from pDC2-mCherry_MCS. Primers included complementary overhangs such that all 3 fragments could then be finally assembled into the linearised pDC2-mCherryT2ABSD_MCS backbone in a single step using an InFusion HD Cloning Kit (Clontech), generating pDC2-mCherryT2ABSD_wtSUB1 (WT *SUB1*) (Figure 5.14). The 1000 bp of native promoter region of *SUB1* used to drive expression from this episomal construct consisted of approximately 1/3 of the ~3000 bp of sequence between the *SUB1* start codon and the start codon of the putative upstream gene which is transcribed in the opposite direction.

For episomal expression of an EXP1mCherry fusion protein (to fluorescently label the PVM), a synthetic cDNA fragment encoding mCherry fused to the C-terminus of the *P. falciparum* *EXP1* gene (PlasmoDB IDPF3D7_1121600) via a polyglycine-alanine linker (GAGGGGGGGGA) was obtained from Geneart. This was subcloned into vector pCR-Blunt using the ZeroBlunt PCR cloning kit (Invitrogen). The resulting plasmid was digested with *AvrII* and *XhoI* before ligating the EXP1mCherry fragment into pDC-mCherry-MCS, digested with the same enzymes, in the place of the mCherry ORF, generating plasmid pDC2-EXP1-mCherry.

3.4 Immunochemical techniques

3.4.1 SDS PAGE

Proteins were fractionated by electrophoresis on 4-15% Mini-PROTEAN TGX Precast protein gels at 200V in Tris/Glycine/SDS buffer (25 mM Tris, 190mM glycine, 0.1% (w/v) SDS).

3.4.2 Western blot analysis

SDS PAGE fractionated protein lysates were blotted onto nitrocellulose membranes in a wet-transfer apparatus overnight using western blot transfer buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol). Membranes were blocked in 5% (w/v) milk powder (Sigma) solution in 0.05% (V/V) Tween20 in PBS (PBST) for 1 h. Membranes were washed three

times for 5 min in PBST before being probed with primary antibodies at 1:1,000 dilution in 1% (w/v) bovine serum albumin (BSA) (Sigma) in PBST (BSA/PBST) for 1 h. Membranes were washed three times for 5 min in PBST before probing with horseradish peroxidase (HRP) conjugated secondary antibodies specific for human, rabbit or mouse IgG (Invitrogen) at 1:10,000 dilution in BSA/PBST 1 h. For detection of triple HA-tagged SUB1, the rat monoclonal anti-HA antibody 3F10 (Sigma) was used at a 1:1,000 dilution, followed by biotin-conjugated anti-rat antibody (Roche) at 1:8,000 dilution, then HRP conjugated streptavidin (Sigma) at 1:2,000 with three washes for 5 min in PBST in-between each step as above. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used according to the manufacturer's instructions, and blots were visualised and documented using a ChemiDoc Imager (Bio-Rad) with Image Lab software (BioRad).

3.4.3 Stripping nitrocellulose membranes of antibody and re-probing with antibody

To strip nitrocellulose western blot membranes of bound antibody in order to re-probe them with fresh antibody, blots were washed three times for 5 min in 5% (v/v) acetic acid in distilled water. Blots were then washed three times for 5 min in PBS before re-blocking in milk powder solution and re-probing with antibody as above.

3.4.4 Immunofluorescence analysis (IFA)

For IFA, air-dried thin films of parasite cultures were fixed in fresh 4% (v/v) paraformaldehyde in PBS for 30 min. Slides were washed twice for 5 min in PBS before cell membranes were permeabilised in 0.1% (v/v) triton X100 (Sigma) in PBS for 10 min. Slides were then washed twice for 5 min in PBS and then blocked in blocking solution (3% (w/v) BSA (Sigma) in PBS) for 1 h before being probed with relevant primary antibodies at 1:500 dilution in blocking solution for 1 h. Slides were washed three times for 5 min in PBS before secondary Alexafluor 488-, 594- or 624-conjugated antibodies specific for human, rabbit or mouse IgG (Invitrogen) at 1:1,000. For detection of triple HA tagged SUB1 a three step protocol was used: rat monoclonal anti-HA antibody 3F10 (Sigma) was used at a 1:500 dilution, followed by biotin-conjugated anti-rat antibody (Roche) at 1:500 dilution, then streptavidin conjugated Alexafluor 594 (Sigma) at 1:1,000 with three washes in between each step as above. Slides were washed three times for 5 min before being mounted in Vectashield antifade mounting medium with DAPI (Vector Laboratories). Images were acquired using a Nikon Eclipse Ni-E widefield upright microscope fitted with a Hamamatsu C11440 digital camera and Nikon N Plan Apo λ 100x/1.45NA oil immersion objective (Nikon, Japan) and NIS Elements software (Nikon), using identical exposure conditions for each wavelength for all samples being compared.

3.4.5 Reagents and antibodies

Monoclonal antibody (mAb) SB-SP1 specific for human erythrocytic spectrin was from Sigma, whilst mAb 89.1, which recognises *P. falciparum* MSP1, has been described previously [Holder and Freeman, 1982], as have rabbit polyclonal antisera against *P. falciparum*.

parum SERA5 [C. R. Collins, Hackett, et al. 2017], SERA6 [Ruecker et al. 2012; Withers-Martinez, Saldanha, et al. 2002] and AMA1 [C. R. Collins, Withers-Martinez, et al. 2009].

3.5 Timelapse videomicroscopy

Viewing chambers (internal volume $\sim 80 \mu\text{l}$) for observation of live schizonts were constructed as described [C. R. Collins, S. Das, et al. 2013; S. Das et al. 2015] by adhering 22 x 64 mm borosilicate glass coverslips to microscope slides with strips of double-sided tape, leaving 2 \sim 2 mm gaps at diagonally opposite ends. Mature Percoll enriched schizonts were incubated for 4-6 h at 37°C in complete medium supplemented with compound 2 (1 μM). Then $\sim 5 \times 10^7$ schizonts were rapidly washed twice in 1 ml of gassed pre-warmed complete medium lacking compound 2, pelleting at 1,800 x g for 1 min. The cells were then suspended in 50 μl of complete medium and introduced into the pre-warmed viewing chamber on a temperature-controlled microscope stage held at 37° C on a Nikon Eclipse Ni-E widefield upright microscope fitted with a Hamamatsu C11440 digital camera and Nikon N Plan Apo $\lambda 100\times/1.45\text{NA}$ oil immersion objective. Images (DIC alone or simultaneous DIC and fluorescence) were taken at 5-10 s intervals over a total of 20-30 min, then annotated and exported as TIFFs or .mov files using the Fiji distribution of ImageJ [Schindelin et al. 2012]. Alexa Fluor 488 phalloidin and Alexa Fluor 647-conjugated wheat germ agglutinin were from ThermoFisher. Phalloidin (Thermo Fisher) was used at 132 nM and wheat germ agglutinin (Thermo Fisher) at 1 $\mu\text{g/ml}$.

3.6 Electron microscopy

3.6.1 Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out in collaboration with Gema Vizcay (laboratory of Roland Fleck). Schizonts were fixed in 2.5% glutaraldehyde at room temperature, washed in PBS, osmicated (1% OsO₄ for 16 hours), dehydrated, critical point dried and sputter coated with 5 nm gold for scanning EM. Images were collected on a JEOL JSM 7610F with 2.6 kV accelerating voltage.

3.6.2 Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out in collaboration with Victoria Hale, Claudine Bisson and Trishant Umrekar (laboratory of Helen Saibil). Schizonts were pelleted by centrifugation, mixed with 20% (w/v) dextran in complete medium containing baker's yeast, then frozen using a HPM100 high-pressure freezer (Leica). Vitrified cells were freeze-substituted using a EM AFS2 (Leica) into Lowicryl HM20 resin (EMS) with 0.2% (w/v) uranyl acetate and cut into 120 nm sections using a UC7 microtome (Leica). Sections were placed on glow-discharged carbon-coated copper London Finder grids (EMS) with 10 nm Protein A-Au fiducials (EMS) and post-stained with 0.2% (w/v) uranyl acetate 4% (w/v) lead citrate. Images and tomograms were recorded using a Model 2040

dual-axis tomography holder (Fischione Instruments) on a Tecnai F20 200 kV field emission gun electron microscope (FEI), equipped with a DE20 camera (Direct Electron). Dual-axis tilt series were acquired at 4.07 Å/pixel from -60 to +60 with an increment of 2 using SerialEM32 and processed using IMOD33 with nonlinear anisotropic diffusion filtering.

3.7 Statistical analysis

Linear regression analysis by analysis of covariance and t-tests were performed using GraphPad Prism 7 software (CA, USA). χ^2 tests were performed manually in Excel (Microsoft). For χ^2 tests, where expected values were less than 5, data from that category was pooled with the adjacent category to avoid confounding results with large percentage differences that arise when numbers are small. A p-value of <0.05 was considered statistically significant.

Table 3.1: **List of oligonucleotide primer sequences used in this study.** Numbers in brackets are primer code numbers of primers used for diagnostic PCR referred to in figures throughout this thesis

Name	Nucleotide sequence
JT111-1p (1)	GATTGTTAGCGAATTACGATTCCTAG
JT111-3p (2)	GATGACTACAATTACTTGCAGATAC
JT111-2p (3)	GAACAGTTCGACGCAGACACGAAGAAC
S65'UTRb-2 (4)	CAGAAAAAGTAAAAGACCAAATGATA
S6EndoEx2Rev (5)	CTTCAGAACATTTATTTTGAAGTTCC
SERA6-5'UTRb (6)	AAAAGTAAAAGACCAAATGATA
SERA6-37 (7)	AAGTAGGAGTCGGACTTAGAA
+27 (8)	CAATATCATTGTAATCAAACAGTGGT
-25 (9)	CCATTGGACTAGAACCTTCAT
-11 (10)	CTTTGCCATCCAGGCTGTTT
JT111-6p (11)	GAGTACCTGCAAAGAAAGGGTATCTTG
JTPbDT3'R (12)	TTACAGTTATAAATACAATCAATTGG
JTS5synthF (13)	GAATGCTATTTCTGCTACGTG
SERA6-34 (14)	GTCCTGGAAGAAGAACGTTGCCGCCGAGACACAACACTGACTTCATG
hsp863'R1 (15)	GACTTTACTGAGACATG
JTPrA1 (16)	GGTTACGGGATCATGTGGG
S6synthRev2 (17)	GTTCAAAACGTTGTGAGTGATG
S65UTRb-2	CAGAAAAAGTAAAAGACCAAATGATA
S6EndoEx2Rev	CTTCAGAACATTTATTTTGAAGTTCC
SERA6-37	AAGTAGGAGTCGGACTTAGAA
JT-S1CO-F	GAATATAGTGGTATTTTAATTCTTCTGTCGAGTACCTGCAAAGAAAGGG
JT-S1syth-R	AGTCTAACTAGTCTCGAGGTGCAGGTATCTGGACTTCTTCTT
JT-S1endo-F	TAAGTTCCATGGGTAAACAACATAGGAGGGAATGAGGTAGATGC
JT-S1CO-R	CCCTTTCTTTGCAGTACTCGACAGAAGAATTAATAATACCACTATATTC
PfSUB1-synth-for-infu	CGGCTAGCCGATTACTGCATGGCCATATAAATGTTACAAATTC
PfSUB1-synth-rev-infu	GGCATCTACCTCATTCCCTCCTATG
PbDT3UTR-for_infu	GAGGGATATGGCAGCTTAATGTTC
PbDT3UTR-rev_infu	CCCCCGGGTCGATACGCCTACCCTGAAGAAGAAAAGTCC

Chapter 4

Results I: A simple and robust plaque assay can be used to assess growth of *P. falciparum* at the clonal level.

As outlined in the Introduction, plaque assays can greatly facilitate the analysis of the effects of drugs, gene modifications and antibodies on the proliferation of intracellular pathogens. The work presented in this chapter describes the optimisation and application of a simple plaque assay that is likely to become an attractive and widely used addition to the available repertoire of malaria research tools. This work was borne out of a personal observation of Arnault Graindorge which was communicated to members of the laboratory of Michael Blackman by Paco Pino (laboratory of Dominique Soldati): that zones of clearance are sometimes seen in erythrocyte layers in cultures of *P. falciparum*. It was serendipitous that this approach proved such a useful tool during attempts to characterise the phenotype of *SERA6* null mutants presented in Chapter 5. Herein is presented the work I performed to develop, optimise and apply the plaque assay. The results were published in the journal PLoS One [Thomas et al. 2016].

4.1 Growth of *P. falciparum* in static erythrocyte cultures in microplate wells produces plaques.

Initially, work was conducted to determine whether asexual blood stage cultures of *P. falciparum* reliably produce plaques in static conditions in microplate wells. To this end, parasite cultures were dispensed in complete medium into the central 60 wells of flat bottomed 96-well microplates (200 µl per well). These were incubated at 37°C in gassed chambers, without disturbing the erythrocyte layers or replacing the medium, and examined periodically over the course of several days using an inverted light microscope. This revealed the gradual appearance and expansion of roughly circular clearances in the otherwise homogenous erythrocyte layers coating the bottom of each well (Figure 4.1). These discontinuities in the erythrocyte layers are referred to as plaques. Henceforth, all work in

this chapter was performed on *P. falciparum* unless otherwise stated (*P. knowlesi* was also used) and all work was performed in flat-bottomed 96-well microplates, simply referred to as microplates.

Initial attempts to document these plaques using inverted microscopes with cameras proved cumbersome as only proportions of the plates, or even only proportions of individual wells could be documented per image. In the search for a more convenient and higher throughput means of documenting plaques microplates were scanned using an Epson Perfection V750 Pro high-resolution scanner in transmission light mode at 4,800 dpi as has been described previously [Sullivan et al. 2012]. It was found that plaques were readily observable in scans of plates. The extremely wide field of view of a scanner is a great benefit as it obviates the need for laborious microscopic examination of individual wells and allows for simple documentation of up to three plates at a time. The cost of a document scanner is also considerably less than an inverted microscope, facilitating the implementation and use of this method, especially in resource poor settings. The cell counter plugin of the free open source software Fiji (<https://imagej.net/Fiji/Downloads>) was used to count plaques on scanned images. It was found that the green channels of the images (obtained by separating the colour channels in Fiji using the 'split channels' tool) provided the greatest contrast between plaques and the erythrocyte layer. The red channel provided the least contrast, with plaques practically undetectable. The clarity of the green channel presumably results from absorbance of green wavelengths of light by the haemoglobin of the erythrocyte layer, while it is transmitted through the area of clearance of erythrocytes that constitutes plaques.

To formally demonstrate that plaque formation was a result of parasite replication, highly synchronous ring stage parasite cultures (approximately 10% parasitaemia, 0.75% haematocrit) were serially diluted (10-fold) into a suspension of uninfected erythrocytes (0.75% haematocrit) in complete medium. Aliquots of each dilution were dispensed across multiple wells of a microplate. Examination of plaques in each well revealed that mean plaque number per well at any given dilution correlated linearly with starting parasitaemia, with no plaques forming in the absence of parasites (Figure 4.2). Similar data was collected from a further two independent experiments, this time with two-fold serial dilutions, with identical results (Figure 4.3). This demonstrated that under the correct conditions parasite growth was capable of producing plaques in erythrocyte layers. These presumably result from sequential rounds of egress and preferential invasion of fresh erythrocytes immediately adjacent to the infected cells such that a 'wave' of erythrocyte destruction emanates outwards from the focus of infection.

To optimise conditions for plaque formation and detection, plaque formation was examined in microplate wells seeded with cultures of a range of haematocrit values: 0.5%, 0.75% and 1%. This revealed that a haematocrit of 0.75% resulted in optimal plaque clarity (Figure 4.4). Interestingly, and unexpectedly, it was noticed that erythrocyte longevity under the conditions used for this assay was also haematocrit dependent. Signs of erythrocyte lysis were observed as early as 10 days in cultures of 0.5% haematocrit, with free haemoglobin appearing in the culture medium and changes in the appearance and homogeneity of the erythrocyte layers. This resulted in plaques with reduced clarity and less distinct perimeters. In contrast, at higher haematocrits erythrocytes were reproducibly

stable for at least two weeks. As the optimum clarity and stability of plaques was found to be achieved when using a haematocrit of 0.75%, this was used for all subsequent work. It is important to note that haematocrits being discussed here depend on dispensing 200 μ l of culture into each well as it is the absolute number of erythrocytes dispensed into a well that determines the thickness of the layer formed at the bottom of a well. Haematocrits were counted precisely using a haemocytometer and were found to be 1.42×10^8 / ml for 0.75% haematocrit cultures. Therefore the number of erythrocyte per well was 2.84×10^7 . Should a smaller volume be dispensed into wells the haematocrit of the culture dispensed must increase accordingly to maintain the same number of erythrocytes in each well.

Plaques typically reached a diameter of 0.1 mm before eventual complete lysis of all erythrocytes between 16-21 days. This was not due to excessive parasitaemia in the microwell cultures; microscopic examination of Giemsa-stained thin smears of cultures recovered from wells containing 30 plaques at 14 days revealed parasitaemia values to be $\sim 0.06\%$ (1 parasite in 1,600 erythrocytes examined), well below what is routinely achieved in standard conditions in flasks.

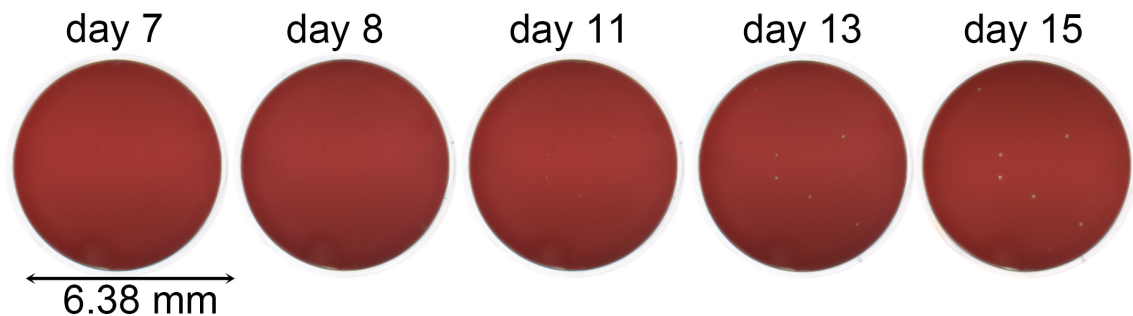


Figure 4.1: **Time-dependency of plaque formation in a static culture of asexual blood stage *P. falciparum* in a microplate well.** Scanned images (RGB channels) of a single microplate well (diameter 6.38 mm) taken at the indicated days following the introduction of a low parasitaemia *P. falciparum* 3D7 clone culture (200 μ l per well, 0.75% haematocrit, starting parasitaemia 0.00004%, corresponding to ~ 6 infected cells per well) showing the time-dependent evolution of plaques. Plaques first became detectable by light microscopy or high-resolution scanning at day 8.

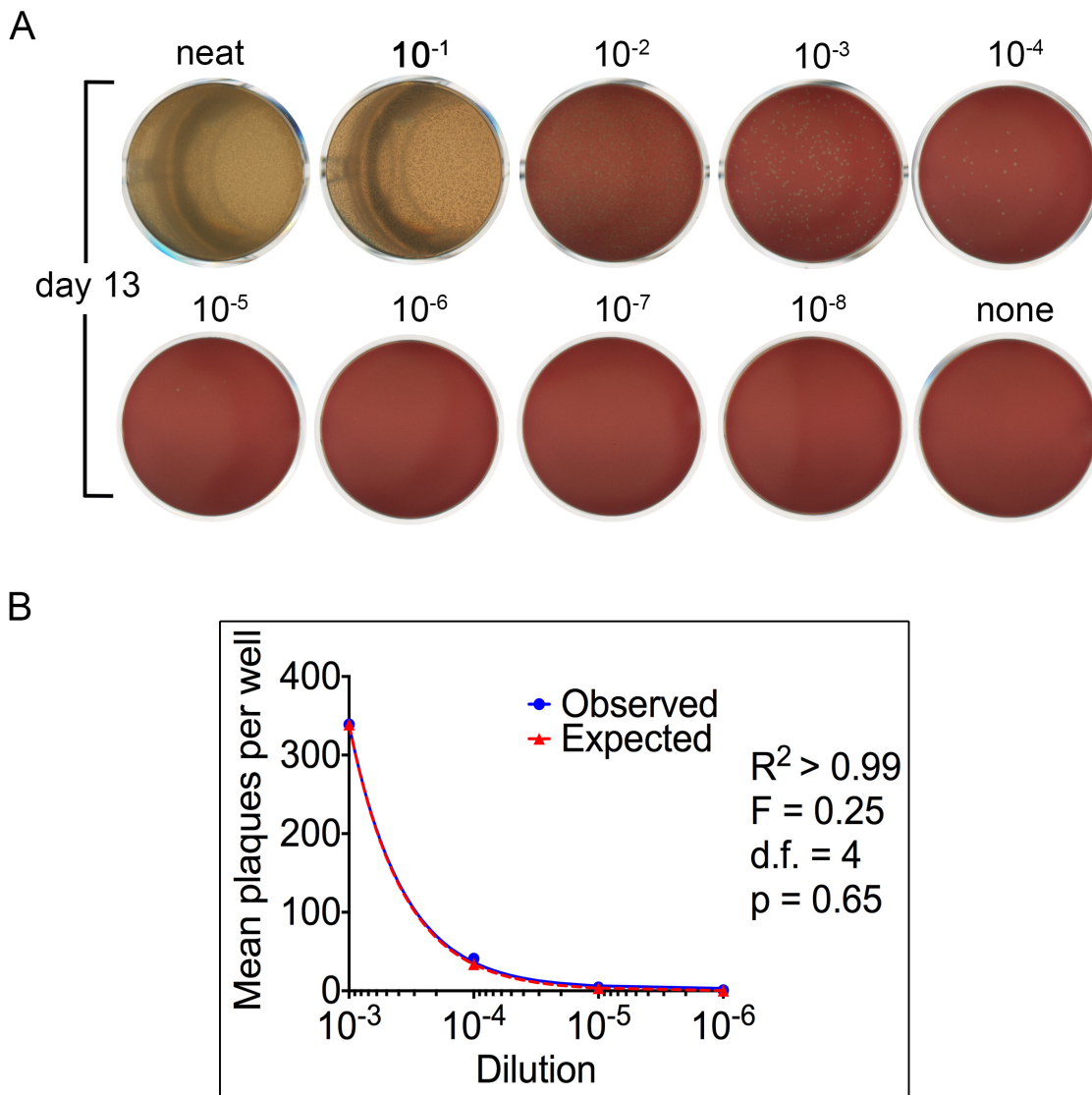


Figure 4.2: Plaque frequency correlates linearly with starting parasitaemia. A) Microplate wells containing 10-fold serial dilutions (dilution of starting culture indicated) of parasite culture (0.75% haematocrit, starting parasitaemia ~13%). Plates were imaged on day 13 following the introduction of the culture into the plate. By this point, at the highest parasite densities (neat and 10⁻¹ dilution) the erythrocyte layer was completely destroyed, whilst discrete plaques were visible at the lower parasite densities. B) A plot showing the mean plaque number per well across 6 replicate wells on a single plate containing 10-fold serially diluted parasite culture. Plaques were only enumerated in wells containing culture diluted between 10⁻³ and 10⁻⁸ as well as in those containing no parasites (none). The wells containing culture at 10⁻² possessed too many plaques to confidently discern discrete plaques. Total plaque numbers across replicate wells were: 2,034 (10⁻³), 245 (10⁻⁴), 26 (10⁻⁵), 4 (10⁻⁶), and 0 plaques (10⁻⁷ and 10⁻⁸). No plaques were detected in wells containing cultures lacking parasites (none). A plot of the observed plaque frequency against dilution (blue) from the experiment is shown alongside a plot of the plaque frequencies expected if there is a linear inverse correlation between mean plaque number and dilution (red). Linear regression by analysis of covariance (ANCOVA) indicated a strong linear inverse correlation between dilution and observed mean plaque density in the wells containing the 10⁻³-10⁻⁶ dilutions. These are not significantly different from the expected values. Values of the statistical data (R^2 , F statistic, number of degrees of freedom (d.f.) and p-value) are shown.

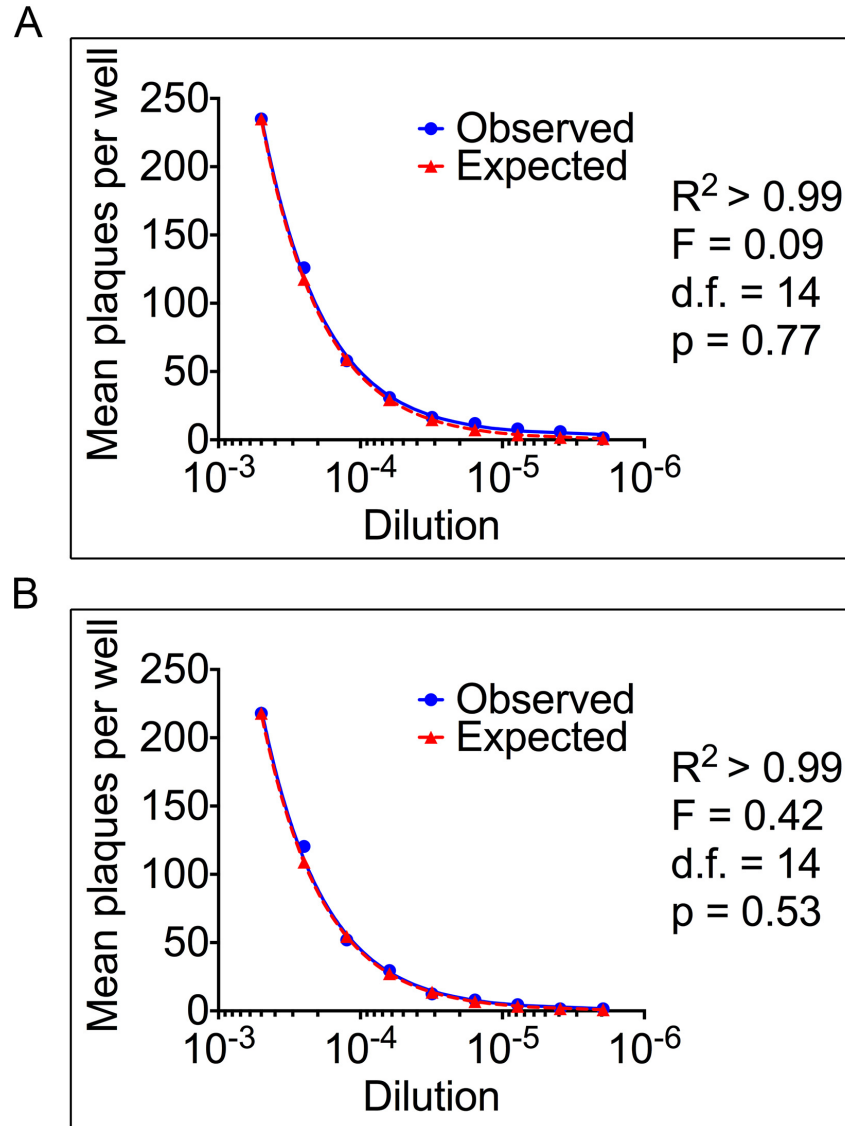


Figure 4.3: **Replicate experiments demonstrate that plaque frequency correlates linearly with parasite number.** Depicted are plaque assay data from two independent experiments (A and B) carried out on different days in which, similarly to the experiment depicted in Figure 4.2, a parasite culture was serially diluted (2-fold) across a microplate. Plaques were enumerated at 14 days. Plots of the observed plaque frequency against dilution (blue) are shown alongside a plot of the plaque frequencies expected if there is a linear inverse correlation between plaque number and dilution (red). Linear regression by analysis of covariance (ANCOVA) indicated strong linear inverse correlations between dilution and observed mean plaque densities in both cases. These are not significantly different from the expected values. Values of the statistical data (R^2 , F statistic, number of degrees of freedom (d.f.) and p-value) are shown.

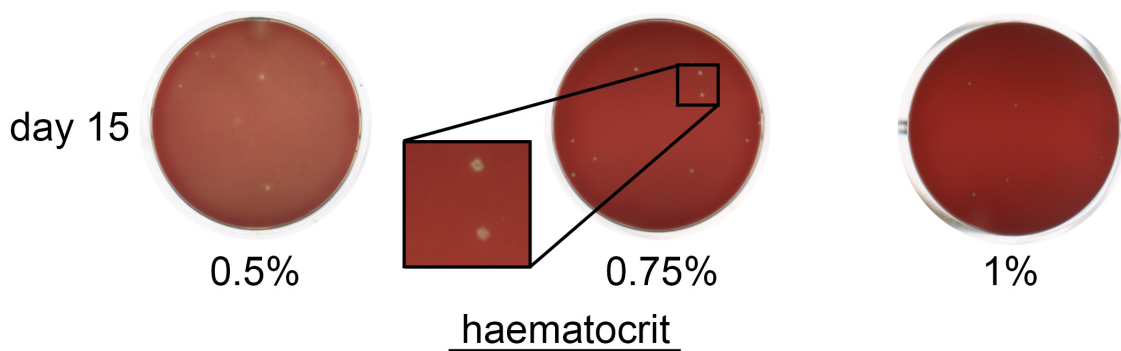


Figure 4.4: **Optimisation of culture haematocrit for plaque formation.** Microplate wells containing cultures at $\sim 0.00004\%$ parasitaemia at the indicated haematocrits, imaged on day 15. Whilst plaque formation was easily detected in the 0.75% haematocrit wells, they were much more difficult to detect at 1% haematocrit, whilst at lower haematocrit values the plaques were typically more diffuse and signs of erythrocyte lysis were observed. Inset, zoomed region of the 0.75% haematocrit erythrocyte layer, showing the discrete nature of the plaques.

4.2 Each *P. falciparum* infected erythrocyte in the starting population forms one plaque.

During parasite growth under static conditions in a microplate well it is possible that diffusive movement of free merozoites, or even infected erythrocytes, might result in the formation of multiple plaques from a single parental infected cell. To investigate whether this is the case, or whether each infected cell results in only one plaque, a synchronous ring-stage culture was diluted into a suspension of fresh erythrocytes at 0.75% haematocrit to obtain a theoretical density of <5 infected erythrocytes per ml of culture. This culture was then dispensed into the central 60 wells of four microplates (200 μ l per well, 240 wells total), and the plaques per well were enumerated 15 days later. If the parasitised cells assort randomly, and if each infected erythrocyte results in a single discrete plaque, the distribution of plaques across the wells should follow Poisson statistics. The experimentally determined frequency of plaques per well was compared to the theoretical, expected frequency, according to a Poisson distribution with the same mean frequency of plaques per well (0.388) as observed in this experiment (Figure 4.5). A χ^2 goodness-of-fit test showed that the observed and expected distributions of plaque frequency were not significantly different from each other (see Figure 4.5). Similar data were obtained from three additional, independent experiments, with widely varying mean frequencies of plaques per well (Figure 4.6); in every case the observed frequency closely matched the expected frequency of plaques per well. Chi-squared tests showed that the observed and expected distributions were not significantly different from each other.

Together with the observation that plaque number correlates linearly with parasite dilution, these results convincingly confirmed that, at least under limiting dilution conditions where plaque number can be accurately determined, each plaque usually derives from a single precursor parasite infected erythrocyte, and that multiple plaques do not frequently arise from a single infected erythrocyte.

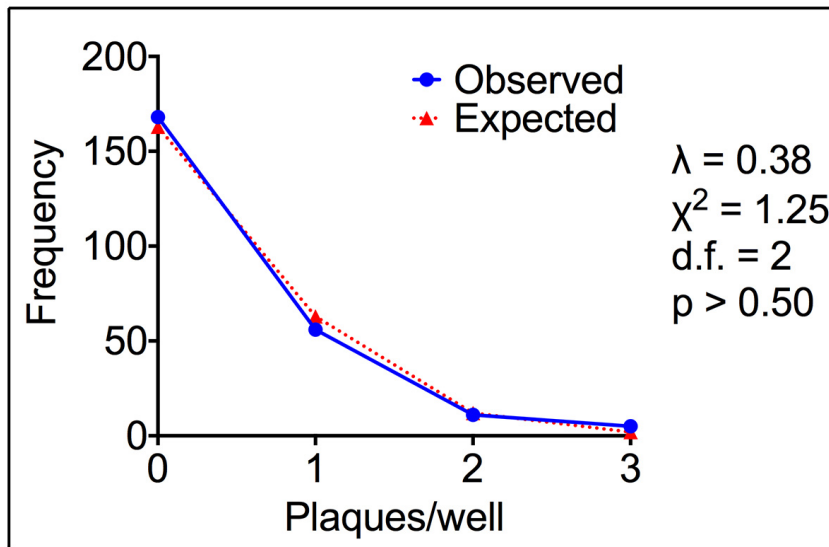


Figure 4.5: **The experimentally observed frequency distribution of plaques per well follows a Poisson distribution.** A parasite culture was dispensed into the central 60 wells of four microplates at a limiting dilution. Plaques were enumerated at 14 days. The experimentally determined mean number of plaques per well (λ) was 0.388. Experimentally observed plaque frequency values are shown (blue) alongside the expected frequency values of a Poisson distribution with a mean of 0.388 (red). A χ^2 goodness-of-fit test showed no significant deviation of the observed distribution from the expected distribution. χ^2 value, number of degrees of freedom (d.f.) and p-value are indicated. For the purpose of statistical analysis, the 3 plaques/well category was pooled with the 2 plaques/well category as the expected frequency value for 3 plaques per well (2), is less than 5. This is to avoid skewing the analysis with large percentage differences that occur by chance between small numbers.

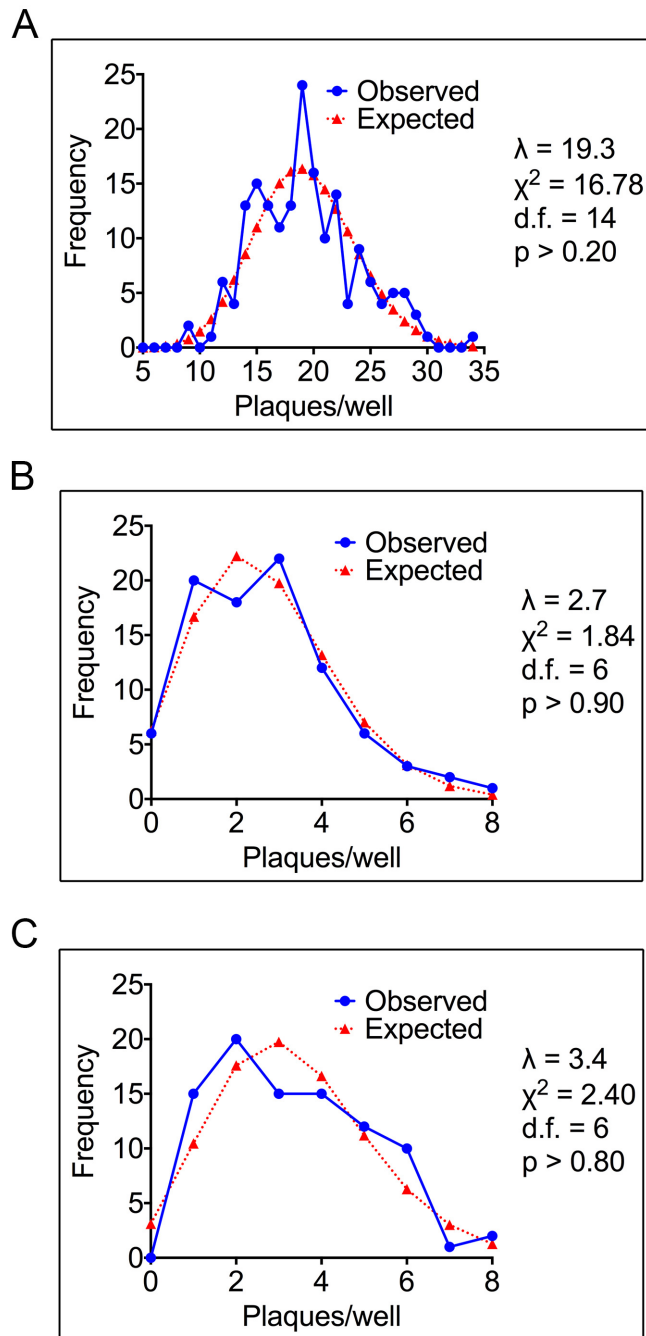


Figure 4.6: **Replicate experiments confirm that the experimentally observed frequency distributions of plaques per well follows a Poisson distribution.** Depicted are data from three independent experiments (A, B and C) carried out as in Figure 4.5 on different days in which a parasite culture was dispensed into microplates. Plaques were enumerated at 14 days and mean number of plaques per well (λ) calculated. Experimentally observed plaque frequency values are shown (blue) alongside the expected frequency values of Poisson distributions with the same mean (red). Chi-squared goodness-of-fit tests showed no significant deviation of observed distributions from the expected distributions. χ^2 values, degrees of freedom (d.f.) and p-values are indicated in each case. Note that for the purpose of statistical analysis, those categories where expected frequency values were less than 5 were pooled to create a single category in order not to skew the analysis with large percentage differences that occur by chance between small numbers.

4.3 Use of the plaque assay for phenotypic analysis of a replication-defective *P. falciparum* mutant.

To explore the broader utility of the plaque assay it was applied to the phenotypic characterisation of a genetically modified clone of *P. falciparum* known to display a partially defined growth defect. *Plasmodium* merozoites are uniformly coated in the GPI-anchored protein MSP1. It has been shown previously that conditional genetic truncation of the *MSP1* gene in *P. falciparum*, such that the C-terminal membrane anchoring domain is removed, results in merozoites lacking surface-bound MSP1 [S. Das et al. 2015]. These mutant parasite egress inefficiently from the host erythrocyte, which results in a replication defect under static growth conditions *in vitro*. The replication rate of parasites lacking surface-bound MSP1 was reduced more than two-fold compared to control parasites [S. Das et al. 2015]. It was predicted that this growth defect should manifest in the plaque assay as a reduction in the number as well as size of plaques. To test this hypothesis, a culture of highly synchronous ring-stage *P. falciparum* MSP1:loxPint parasites [S. Das et al. 2015; Jones et al. 2016] which possess a floxed 3' portion of the *MSP1* gene, and constitutively express the DiCre fragments [C. R. Collins, S. Das, et al. 2013], was divided into two and mock-treated or RAP-treated (to induce DiCre mediated gene excision and thus disruption of MSP1 function). These cultures were then diluted identically to a theoretical density of ~75 parasitised erythrocytes per ml (15 parasites per well, 0.75 haematocrit) and dispensed into microplate wells. The resulting plaques were documented and quantified 14 days later with the pixel area of individual plaques determined using the Magic Wand tool of Adobe Photoshop CS5. Plaques that formed in RAP-treated cultures were smaller and approximately five times less numerous (mean plaque area (pixels) = 199.3, SD = 9.2, n = 54) than those in the mock-treated cultures (mean plaque area (pixels) = 329.1, s.d. = 6.7, n = 205) with a two-tailed independent t-test revealing this difference to be highly significant ($t = 9.290$, d.f. = 257, $p < 0.0001$) (Figure 4.7). The differences in mean plaque area values indicate a fitness defect associated with truncation of MSP1, consistent with the previously observed ~2-fold slower replication rate displayed by RAP-treated MSP1:loxPint parasites, compared to mock-treated controls in standard parasite replication assays [S. Das et al. 2015]. These results showed that the plaque assay can be used as a simple means of assaying and characterising even rather subtle effects on parasite fitness resulting from mutagenesis.

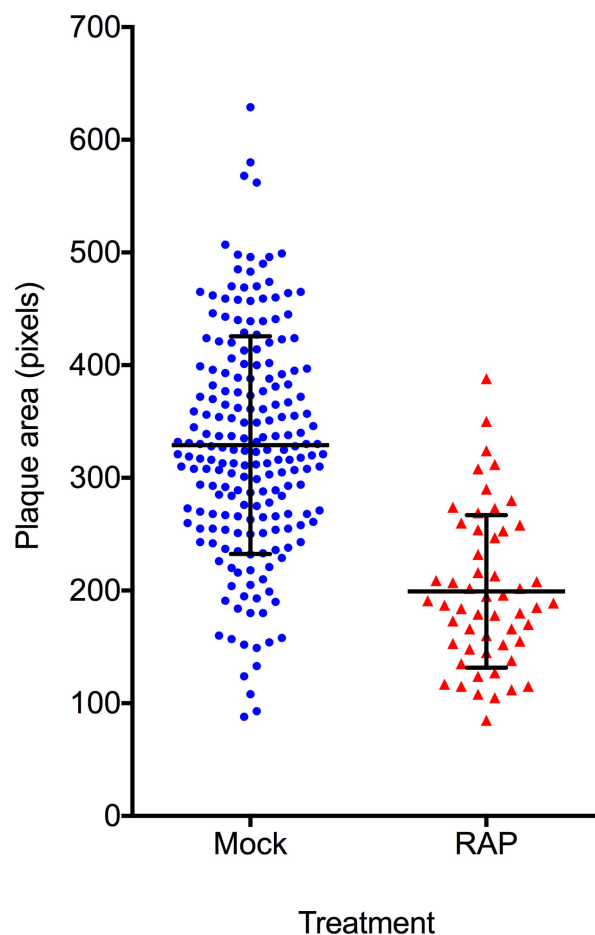


Figure 4.7: **Phenotypic characterisation of a replication-defective *MSP1* mutant using the plaque assay.** Scatter plots showing the distribution of plaque areas (pixels) obtained following mock- or RAP-treatment of *MSP1:loxPint* parasites. Plaque numbers were $n = 205$ for mock-treated samples and $n = 54$ for RAP-treated samples. Plaque areas were quantified using the Magic Wand tool of Photoshop CS5 (Adobe). Horizontal bars indicate mean plaque area ± 1 standard deviation

4.4 Rapid phenotypic analysis of a conditional lethal *Plasmodium falciparum* mutant.

To further examine the utility of the plaque assay it was used to characterise the replication defect of another *P. falciparum* mutant deriving from work presented in this thesis. As presented in Chapter 5, a transgenic clone of *P. falciparum* was produced called *SERA6:loxP* in which the entire *SERA6* open reading frame and promoter region were flanked by *loxP* sites. As is presented in Section 5.3, RAP-mediated excision of the *SERA6* gene results in a lethal phenotype. It was predicted that this should result in a significant decrease in plaque formation. A highly synchronous ring-stage culture was divided into two and mock- or RAP-treated as described in Section 5.3. The cultures were then diluted equivalently to a theoretical density of ~ 75 parasites per ml (15 parasites per well) in a 0.75% haematocrit erythrocyte suspension, dispensed into microplates and plaques quantified 14 days later. The results showed that RAP-mediated *SERA6* gene disruption resulted in a substantial reduction in plaques compared to mock-treated samples (Figure 5.10); only a single plaque was seen following RAP-treatment. Whilst RAP-mediated excision in the *P. falciparum* DiCre system is highly regulated and efficient, it is rarely complete [S. Das et al. 2015; C. R. Collins, S. Das, et al. 2013]. This results in heterogeneity in the RAP-treated population, with a small proportion of parasites failing to undergo excision following RAP-treatment. It was therefore predicted that the single plaque that was observed in the RAP-treated culture was produced by a non-excised parasite. To test this hypothesis parasites recovered from the plaque-positive well were expanded in culture and analysed by diagnostic PCR (see Section 5.3.2). This demonstrated that, as predicted, these parasites possessed an intact, non-excised *SERA6* locus (Figure 5.13). The use of the plaque assay therefore allowed the visualisation of parasite fitness at the level of individual parasites. This has allowed an unambiguous assessment of the essentiality of *SERA6* which would not be possible if assays could only be performed on mixed populations containing both excised and non-excised parasites.

4.5 The culture-adapted *P. knowlesi* clone A1.H1 produces irregularly shaped and indistinct plaques.

Until recently it has only been possible to culture *P. knowlesi* *in vitro* using cynomolgus macaque erythrocytes which, given the difficulty in obtaining a regular supply of cynomolgus blood, precludes most laboratories from culturing *P. knowlesi*. The A1.H1 strain of *P. knowlesi* has recently been adapted to grow in human erythrocytes *in vitro* [Moon, J. Hall, et al. 2013]. To determine whether the plaque assay is applicable to examining the growth of *P. knowlesi* *in vitro*, as has been demonstrated for *P. falciparum*, a culture of *P. knowlesi* A1.H1 clone ($\sim 5\%$ parasitaemia, 0.75% haematocrit) was serially diluted (10-fold) into erythrocyte suspensions across a microplate. Microplates wells were examined for plaques every day between 12 and 20 days after dispensation into plates, without changes of culture medium. Plaque formation was observed, correlating linearly with dilution. However these plaques lacked clarity compared to those resulting from growth of

P. falciparum (Figure 4.8). They were also noticeably less round and regular in shape making assessment of number more difficult.

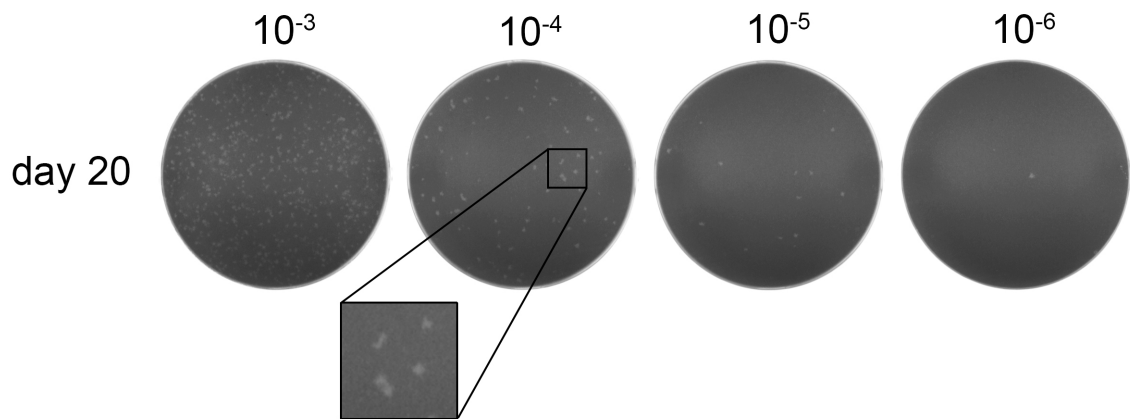


Figure 4.8: ***P. knowlesi* asexual blood stage cultures form plaques that lack clarity and regularity compared to those in cultures of *P. falciparum*.** A) a culture of *P. knowlesi* A1.H1 clone ($\sim 5\%$ parasitaemia, 0.75% haematocrit) was serially diluted (10-fold) across a microplate. Depicted are wells of culture at the indicated dilutions. Scanned images were taken 20 days after dispensing of culture. Inset, zoomed region of a well showing 4 plaques. Only the green channel has been depicted for maximum clarity, and brightness and contrast have been significantly altered for the purposes of depiction.

4.6 Conclusions

There has long been a need for a simple and robust plaque assay suitable for use with *P. falciparum*. Here such a system has been developed, using the simple approach of culturing the parasite under static, limiting dilution conditions in human erythrocytes in flat-bottomed microplates. Visualisation of plaque formation was haematocrit-dependent, but it was found that plaques were easily detectable within ~2 weeks under optimised conditions (0.75% haematocrit). This method with a thicker layer of erythrocytes likely allows productive parasite proliferation and plaque growth in 3 dimensions, as opposed to the two-dimensional plaque expansion allowed by the adherent erythrocyte monolayers used in previously-described *P. falciparum* plaque assays [Bruce et al. 1990; Inselburg 1983; Williams 1999]. The plaques, which presumably consist predominantly of haemoglobin-free erythrocyte ghosts, cell debris and haemozoin pigment, are completely stable under conditions of regular manual plate transferral to and from incubators, even without the use of semi-solid medium, probably because the small volumes and surface areas of the liquid columns in each microplate well limit liquid movement and consequent disturbance of the erythrocyte layer. Use of larger wells or flasks would probably destabilise plaques, which may explain why the formation of these structures has not previously been described. Plaque integrity and stability was further ensured in the assay by not replacing the medium during the 2-3 week period of the assay. Although this may have the effect of limiting parasite growth rates, it was found that up to a plaque density of at least 340 plaques per well, plaque frequency correlated with starting parasite density, implying that, at least within this parasitaemia range, plaque formation is not adversely affected by plaque density. Plaques produced by clonal parasite populations were not completely homogeneous in size or shape, but this is a feature of many plaques, including those produced by *Toxoplasma*. The *P. falciparum* plaques are easily visible using an inverted light microscope and, similar to viral plaques [Sullivan et al. 2012], can be rapidly imaged with minimal disturbance using an inexpensive, commercially available document scanner, avoiding the need for laborious photographic imaging of individual wells. Indeed, using a high resolution (4,800 dpi) flat-bed scanner in transmission light mode, it was possible to scan and document up to 3 plates simultaneously in a single TIFF image, allowing for rapid documentation of multiple plates. The availability, low cost and lack of user training required to use a flat-bed scanner makes this a highly accessible, medium-throughput means of documenting the results of assays.

The *P. falciparum* plaque assay has important advantages compared to those commonly used for viruses and other intracellular pathogens such as *Toxoplasma*. Mature erythrocytes do not replicate, so there is no need to take into account changes in host cell confluency over the course of the assay. Furthermore, there is no requirement to fix and stain the cells before plaque analysis. The ability to easily visualise parasite clonal growth has other advantages. The plaque assay is now regularly used in the laboratories of Michael Blackman, Moritz Treeck and Edgar Deu for rapid limiting dilution cloning of *P. falciparum* lines without the need for laborious determination of starting parasitaemia values, simply by performing serial dilutions of a parasite culture across a plate then picking clones only from those wells that contain a single plaque. It is important to transfer these

clones from the plaque assay plates into cultures containing fresh erythrocytes before the plaque assay cultures begin to undergo generalised erythrocyte lysis, which was found usually occurs between 16-21 days after initiating the plaque assay.

Reverse genetic technologies have become increasingly important in malarial research over the past two decades. With the relatively recent introduction of Cas9-mediated mutagenesis [Wagner et al. 2013; Ghorbal et al. 2014] and conditional gene modification technologies in *P. falciparum* discussed in Section 1.11 the development of simple assays such as that described here is particularly timely. The plaque assay has multiple applications; it can be used to rapidly phenotype genetic mutants without the need for complex equipment or facilities (e.g. flow cytometry), but it will also be invaluable for the calculation of kill-curves with anti-parasite drugs and antibodies, for the selection of drug-resistant or fast-growing mutants, for facile comparison of parasite fitness under a range of growth conditions (e.g. in different defined media) and numerous other uses. Differences in parasite growth rate are determined over the course of the 4-7 cycles required to produce visible plaques, providing some advantages over traditional growth assays in that the cultures do not need to be passaged or fed. Importantly, the assay complements other widely used approaches for quantification of parasite growth, including light microscopy, flow cytometry and assays based on expression of parasite proteins or enzymes [Makler and Hinrichs 1993; Noedl, Wernsdorfer, et al. 2002], incorporation of fluorescent dyes [Ndiaye et al. 2010; J. D. Johnson et al. 2007] or radioactive hypoxanthine [Desjardins et al. 1979]. Although not explored here, the assay may be suitable for scaling up to high throughput image based screens using high content microscopy. Indeed, colleagues in the laboratories of Edgar Deu, and Moritz Treeck now routinely use independently developed Fiji based semi-automated methods of plaque counting to increase the throughput of plaque assay analysis (personal communication, Sophie Ridewood and Hugo Belda).

The plaque assay may be applicable to the study of other *Plasmodium* species that can be cultivated *in vitro*. Adaptations to the protocol by Robert Moon and colleagues have already achieved significant improvements on the results shown here for detection of plaques formed in cultures of *P. knowlesi* (personal communication Robert Moon). It was found that gently aspirating half of the culture medium in each well (100 μ l) and replacing it with fresh medium regularly (every 3 days) significantly improved the clarity of plaques compared to when medium was unchanged (as was performed in the study presented here) despite resulting in some disturbance to some areas of the erythrocyte layers. The improvement in plaque clarity that results from medium changes is likely due to enhanced parasite replication rates. *P. knowlesi* appears to be more sensitive to toxic components of the culture medium that result from nutrient degradation compared to parasite clones derived from *P. falciparum* 3D7 clone (personal communication, Robert Moon). One such source of toxic components is L-glutamine, an essential amino acid present in many culture medium formulations. It is important for parasite growth but its instability at physiological pH causes it to breakdown to form pyroglutamate and ammonium, the latter of which is toxic. The extended period that *P. falciparum* 3D7 clone has been cultured *in vitro* (years, or even decades in some cases) (personal communication Christine Collins) has likely resulted in it becoming highly adapted to such conditions of *in vitro* culture, compared to the *P. knowlesi* A1.H1 clone which was culture adapted only recently. It is possible that, in

the *P. knowlesi* assays performed in the study presented here, parasite replication rates declined over the course of the assay as a result of toxin accumulation. No attempt was made to recover and expand parasite cultures from the wells, or to examine them for the presence of live parasites by Giemsa stained thin smear and so it is possible that the parasites may have died completely by the end of the assay. The use of glutaMAX, a stabilised form of L-glutamine, could help reduce or even eliminate the need for medium changes when performing plaque assay analysis on *P. knowlesi*. Even with the improvements achieved in plaque clarity, *P. knowlesi* plaques appear to still be irregular in shape (personal communication Rob Moon). This may result from the well documented greater extracellular motility and longevity of free *P. knowlesi* merozoites [Bannister et al. 1975; J. Johnson et al. 1980] which may allow dispersal of released merozoites over greater distances within the wells than occurs with *P. falciparum*, and consequently a less constrained plaque perimeter.

One shortcoming of the plaque assay seems to be its relatively low dynamic range or 'sensitivity' in measuring parasite growth. As has been demonstrated [S. Das et al. 2015], RAP-induced disruption of the MSP1 gene of MSP1:loxPint clone parasites results in an approximately 2-fold slower replication rate. In the plaque assay performed in this study this defect manifested as an approximately five-fold reduction in plaque number. As such the plaque assay might be said to be relatively insensitive in reporting the growth of all parasites; presumably, stochastically, some grow too slowly over the course of a plaque assay to visualise plaques such that the readout from the plaque assay 'over-exaggerates' the growth defect. For detailed phenotypic analyses, the plaque assay is therefore unlikely to replace standard flow cytometry-based replication assays. However, it will complement such approaches. Given the ease with which it can be performed, the low degree of expertise required and the lack of any requirement for expensive equipment, the plaque assay will likely prove a valuable addition to the experimental tools available for studying this important pathogen, especially at a time when CRISPR/Cas and Cre recombinase technologies are changing the scope of questions can be addressed with regards to *Plasmodium* biology.

Chapter 5

Results II: SUB1 and SERA6 are members of an essential proteolytic cascade that regulates blood stage egress.

As outlined in the Introduction, SUB1 and SERA6 are hypothesised to play roles in egress during the asexual blood stage cycle of *P. falciparum*. Several previous studies have led to the hypothesis that SUB1 acts as a central regulator of egress, cleaving various substrates in the PV following its release from exonemes shortly before egress. SUB1 has been proposed to both prime merozoites for invasion by cleaving MSP1 on the merozoite surface and achieve their release from the erythrocyte [Yeoh et al. 2007; Koussis et al. 2009]. One of the substrates of SUB1 is the putative papain like cysteine protease SERA6, and previous work has suggested that its cleavage activates a proteolytic function [Ruecker2012a,]. The timing of this makes involvement of SERA6 in egress or invasion likely. In this chapter the work conducted during this PhD project that adds to our understanding of SUB1 and SERA6 function, as well as egress more generally, will be outlined. Work presented in this chapter has recently been published in combination with work of Michele Ser Ying Tan as an advanced online publication in Nature Microbiology (DOI: 10.1038/s41564-018-0111-0).

5.1 DiCre mediated excision of crucial catalytic residues of *SUB1* or the entire *SERA6* coding region results in efficient silencing of gene expression.

All previous attempts to disrupt the *SUB1* [Yeoh et al. 2007] and *SERA6* [S. K. Miller et al. 2002; McCoubrie et al. 2007] genes in *P. falciparum* by integration of episomal constructs by single crossover homologous recombination have proved unsuccessful, suggesting that both are essential for the viability of the asexual blood stage cycle *in vitro*. This approach does not definitively confirm essentiality of a gene however, which requires a conditional system of disruption. With the advent of the DiCre system there existed an

opportunity to disrupt both of these genes for the first time, using a conditional system, to investigate their roles and unambiguously determine their essentiality. Indeed, a recent study has demonstrated that *SERA5*, which could not be disrupted by a non-conditional approach, is in fact not essential when this conditional system is used [C. R. Collins, Hackett, et al. 2017].

To this end, the DiCre system was used to conditionally excise a portion of the *SUB1* gene encoding crucial catalytic residues (Figure 5.1), or the entire *SERA6* coding sequence and promoter (Figure 5.2). To achieve this, *loxP* sites were introduced into the loci in the 1G5DC clone [C. R. Collins, S. Das, et al. 2013] through single crossover homologous recombination of episomal constructs. In the case of *SUB1*, a *loxP* containing heterologous intron (*loxPint*) [Jones et al. 2016; S. Das et al. 2015] was inserted within the catalytic domain, as well as a *loxP* site downstream of the stop codon, such that a portion of the catalytic domain including the active site nucleophile Ser⁶⁰⁶ and the oxyanion hole residue Asn⁵²⁰ were flanked with *loxP* sites ('floxed') (Figure 5.4). In the process, *SUB1* was fused to a C-terminal triple HA tag and the endogenous *SUB1* 3' UTR was replaced with the *PbDT* 3' UTR to regulate gene expression. RAP-induced excision of the sequence between the *loxP* sites was therefore expected to truncate the catalytic domain which in turn was expected to ablate *SUB1* protease activity. In the case of *SERA6*, a single *loxP* site already existed on chromosome 2, upstream of *SERA6* (immediately downstream of the *SERA5* stop codon), in the 1G5DC clone such that integration of a single *loxP* site downstream of the *SERA6* stop codon, was sufficient to flox the entire gene (Figure 5.5). Again, the endogenous 3' UTR was replaced with the *PbDT* 3' UTR.

The integration constructs pHH1_*SUB1*HA3_*loxP* and pHH1_*SERA6*_loxP, for floxing of the *SUB1* and *SERA6* genes respectively, were transfected into 1G5DC clone parasites and 'drug cycling' of cultures performed to enrich for parasites in which integration into the chromosome had occurred. Following three rounds of drug cycling parasite clones were obtained by serial dilution for each gene knockout. The *SUB1* conditional knockout clones were called SUB1HA3:loxP clone 3E4 and 5E11, whilst the *SERA6* conditional knockout clones were called SERA6:loxP clone C4 and D2. Following isolation of these clones gDNA was extracted from cultures and PCR analysis performed to confirm integration of the pHH1_*SUB1*HA3_*loxP* (Figure 5.1) and pHH1_*SERA6*_loxP (Figure 5.2) constructs. The presence of the DiCre expression cassette was also confirmed by PCR analysis in each of the four clones (Figure 5.3) as this locus is liable to revert to a wild-type locus in the 1G5DC clone resulting in loss of expression of the DiCre fragments (personal communication Christine Collins and Sujaan Das).

To determine whether silencing of *SUB1* expression could be achieved following RAP-induced excision, synchronised ring-stage SUB1HA3:loxP clone 3E4 and clone 5E11 parasites (<14 h old) were mock- or RAP-treated for 4 h before being washed and cultured as standard. PCR analysis of gDNA extracted from mature parasites (~48 h old) demonstrated that highly efficient excision had taken place at the *SUB1* locus (Figure 5.6). Western blots of SDS-PAGE fractionated protein lysates (collected in parallel with gDNA samples) were probed with the α -HA monoclonal antibody (mAb) 3F10. The almost complete lack of western blot signal demonstrated that the excision of the *SUB1* locus had resulted in silencing of expression of full length SUB1 by 48 h (Figure 5.6). Analysis of blots with

an α -SUB1 polyclonal rabbit serum [Withers-Martinez, Saldanha, et al. 2002] corroborated this. This antibody revealed a weak ~ 75 kDa band (full length SUB1 is 82 kDa) that likely corresponds to a product expressed from the truncated *SUB1* gene that fails to fold properly, preventing the co-translational auto-proteolytic processing that normally results in the dominant processed form of SUB1 in mature schizonts [Sajid, Withers-Martinez, and Blackman 2000]. Note that this p47 form often runs as an apparently larger species on SDS PAGE gels. IFA analysis of mock- and RAP-treated 3E4 clone parasites using mAb 3F10 further confirmed the loss of *SUB1* expression from the parasite population. 88.2% of mock-treated 3E4 clone schizonts (total of 493 mature schizonts examined) were positive for the mAb 3F10 signal (Figure 5.6). In contrast, in samples of RAP-treated 3E4 clone parasites only 0.2% (10 out of 5,056) mature schizonts examined were positive for 3F10 staining. This suggests that the rate of excision following RAP-treatment in this clone was approximately 99.8%.

SERA6:loxP clone C4 ring-stage parasites were mock- or RAP-treated and cultured as standard. PCR analysis demonstrated excision of the *SERA6* locus by the end of this erythrocytic growth cycle (cycle 0, ~ 48 h) (Figure 5.7). Interestingly, the *SERA6* null (Δ *SERA6*) parasites displayed no phenotype until ~ 96 h post RAP-treatment (i.e. the end of erythrocytic cycle 1) (see Section 5.7), so parasite culture was continued and gDNA was extracted from ~ 96 h old parasites. PCR performed on gDNA extracted at this later time point (end of cycle 1) demonstrated that excision of the *SERA6* locus was readily apparent with little evidence of the non-excised locus. In addition to interrogating the chromosomal architecture of the *SERA6* locus following mock- or RAP-treatment, PCR was also used to assess the presence of the circular DNA plasmid which is expected to be excised from the chromosome following DiCre-mediated excision (Figure 5.5 and Figure 5.7). This revealed that the excised plasmid is readily detectable at 48 h, but by 96 h it is almost undetectable in the population (Figure 5.7). In D2 clone parasites excision of the *SERA6* locus by PCR was performed only at the end of cycle 1 (~ 96 h old), following mock- or RAP-treatment (Figure 5.7). Again, as is evident from the presence of a band corresponding to the excised locus after RAP-treatment, efficient RAP-induced DiCre mediated excision of the *SERA6* locus had occurred.

Southern blot analysis is often performed to confirm integration of episomal constructs into the genome. Given that RAP treatment of SUB1HA3:loxP clones 3E4 and 5E11 and SERA6:loxP clones C4 and D2 clearly resulted in loss of protein expression, as expected, alongside the predicted PCR products, southern blot analysis was not deemed necessary in this study. It is possible that PCR analysis might not reveal some aberrant integration of episomal constructs. For example concatemerisation of episomal constructs might occur followed by integration into the locus of interest as occurred in a study of *AMA1* [Yap et al. 2014]. Was this to occur in the case of the *SUB1* or *SERA6* loci, RAP induced excision would still excise the intervening DNA between the most extreme loxP sites such that the chromosomal architectures of the *SUB1* or *SERA6* loci would be the same following RAP treatment as if only a single copy of the plasmid had integrated - resulting in a knockout. Furthermore Integration of the plasmids elsewhere into the genome for either of the integration plasmids would not result in expression of the *SUB1* or *SERA6* genes as the sequences in the episomal constructs are lacking the extreme 5' regions of the

ORFs including the start codons and signal sequences. Again, the western blot analysis and IFA confirm that expression, including with antibodies raised against the proteins themselves, not just epitope tags, is lost following RAP-induced excision. The genetic complementation outlined in Section 5.4 further confirms that the phenotypes that are observed following loss of *SUB1* or *SERA6* expression presented herein result from loss of these genes rather than any other alteration of the genome.

To examine *SERA6* expression at the end of cycle 0 and cycle 1, western blots of SDS-PAGE fractionated protein lysates from mock- and RAP-treated *SERA6:loxP* clone C4 and clone D2 were probed with either an α -*SERA6* mAb specific for the N-terminus of the protein (3F12.1C9) or a rabbit serum raised against the *SERA6* putative papain like domain (S6C1). In both cases it was evident that *SERA6* expression was silenced by the end of cycle 1 (~96 h old) (Figure 5.7). A faint band corresponding to full length *SERA6* was detected at 48 h with the S6C1 rabbit serum, suggesting expression was severely diminished but not completely absent at this time point. This likely explains why the Δ *SERA6* parasites were apparently phenotypically indistinguishable from control parasites until 96 h (see Section 5.7). The expression observed up to the end of cycle 0 likely results from the excised plasmid which was shown to be present at 48 h and contains the *SERA6* ORF under control of its native promoter. Indeed it is well documented that continued expression of even low amounts of enzymes can be sufficient to sustain WT phenotypes due to their extreme catalytic efficiencies [Méthot et al. 2007; Methot et al. 2008]. The plasmid, of which there is presumably only one copy per schizont at 48 h, as RAP-treatment was performed on ring-stage parasites which possess only a single copy of the genome, is presumably not replicated and acquired by no more than one daughter merozoite at the end of cycle 0. Following proliferation of the population only a minority of parasites would be predicted to possess this plasmid during cycle 1. It may also be lost due to instability diminishing the number of parasites possessing this plasmid during cycle 1 still further. To assess this possibility, IFA analysis was performed on *SERA6:loxP* clone C4 parasites using both of the α -*SERA6* antibodies, however a low signal to noise ratio made it difficult to reliably determine whether individual parasites were positive or negative for staining. As such the rate of excision could not be determined for the *SERA6:loxP* clones.

Together, these data clearly demonstrate that DiCre mediated gene excision of the *SUB1* and *SERA6* loci occurs rapidly and efficiently following RAP-treatment and in the vast majority of the transgenic parasite population. This resulted in silencing of *SUB1* and *SERA6* expression by the ends of erythrocytic cycle 0 and cycle 1 respectively.

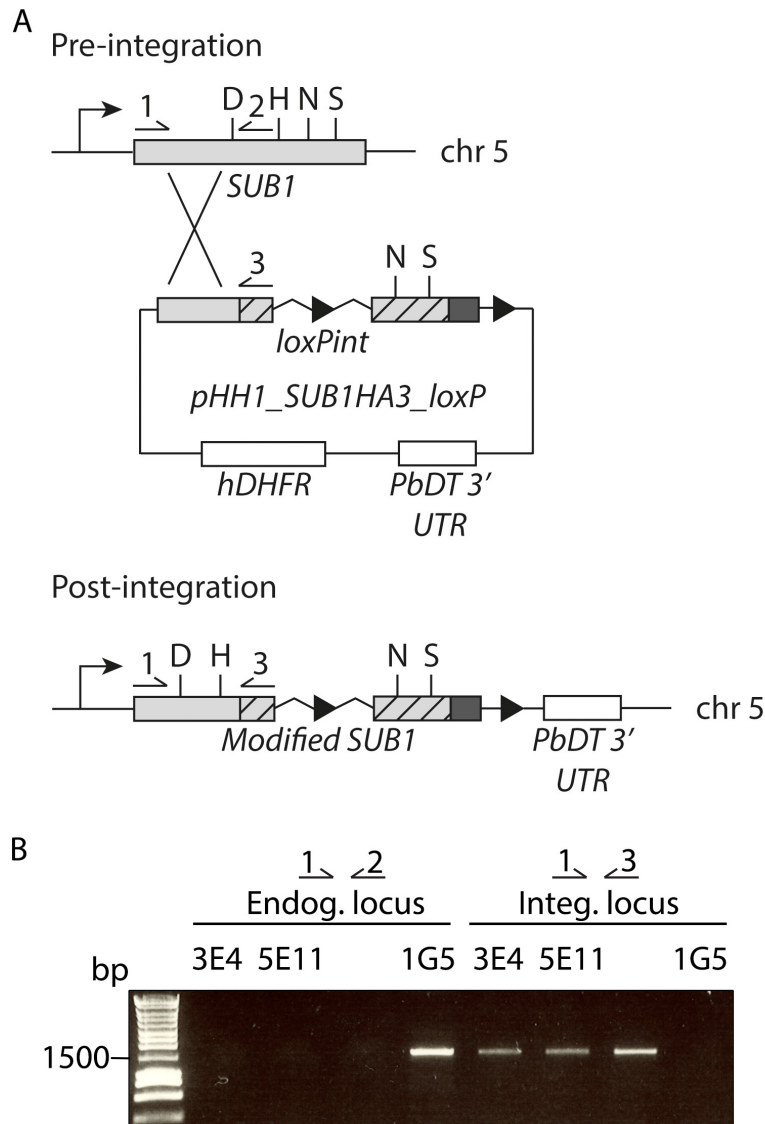


Figure 5.1: Integration of episomal construct pHH1.SUB1HA3.loxP floxes a portion of the *SUB1* gene containing crucial catalytic residues. A) Schematic showing architecture of the *SUB1* locus (chromosome 5) in *P. falciparum* 1G5DC clone prior to integration of construct pHH1.SUB1HA3.loxP and as expected following construct integration. This construct is designed to integrate by single-crossover homologous recombination and contains an artificial *loxP* site (arrowheads) containing intron (*loxPint*) within a recodonised portion of *SUB1* (hatched) and a *loxP* site downstream of the *SUB1* stop codon such that integration will flox a portion of the gene containing crucial catalytic residues. Following integration, a triple-HA tag (HA3) is fused to the C terminus of *SUB1* (dark grey) and the *PbDT* 3' UTR is placed downstream of *SUB1* to regulate gene expression. Human *DHFR* (*hDHFR*) drug resistance marker is shown. Catalytic residues of *SUB1* are indicated (single letter amino acid code): Asp, D; His, H and Ser S. Oxyanion hole residue is indicated: Asn, N. B) PCRs to detect endogenous *SUB1* locus (Endog. locus) and the locus following integration of construct pHH1.SUB1HA3.loxP (Integ. locus) in clones 3E4 and 5E11. The parental 1G5DC clone is included for comparison. Primers used for diagnostic PCR to assess integration are shown (half-arrows) (see Table 3.1 for primer sequences). The endogenous locus is expected to produce a product of 1440 bp, the integrant locus is expected to produce a product of 1445 bp.

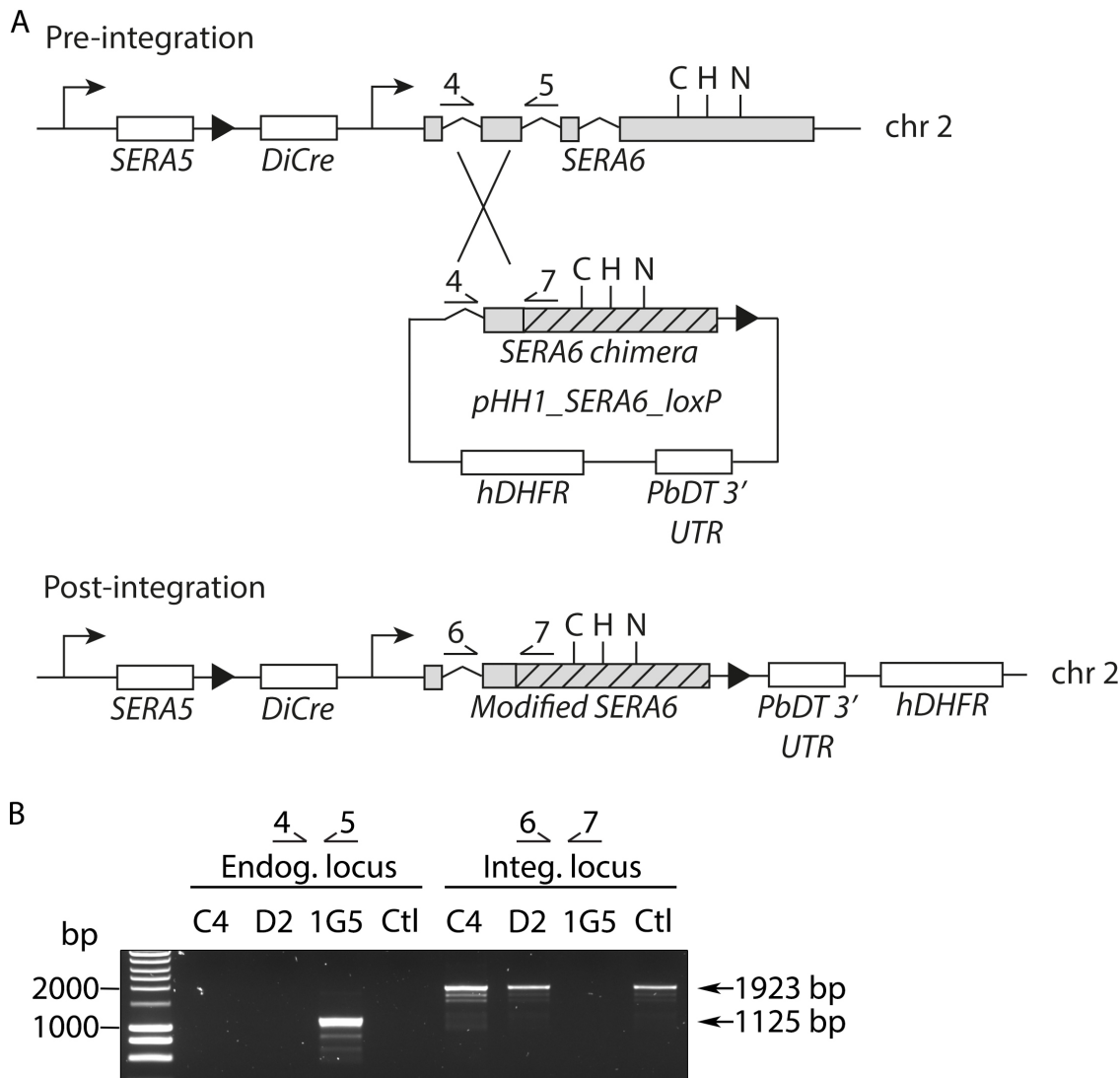


Figure 5.2: Integration of construct pHH1_SERA6_loxP floxes the entire SERA6 ORF. Schematic showing architecture of the *SERA6* locus (chromosome 2) in *P. falciparum* 1G5DC clone prior to integration of construct pHH1_SERA6_loxP and as expected following construct integration. This construct is designed to integrate by single-crossover homologous recombination and introduces a single *loxP* site (arrowhead) downstream of the *SERA6* stop codon such that integration will flox the entire *SERA6* ORF given the prior existence of a single *loxP* site upstream of *SERA6* in the 1G5DC clone. Following integration the *PbDT* 3' UTR is placed downstream of *SERA6* to regulate gene expression. The upstream *SERA5* gene, human *DHFR* (*hDHFR*) drug resistance marker and DiCre expression cassette are shown. The recodonised portion of *SERA6* is shown (hatched). Predicted conserved catalytic triad residues of *SERA6* are indicated (single letter amino acid code); Cys, C; His, H; Asn, N. B) PCRs to detect endogenous *SERA6* locus (Endog. locus) and the locus following integration of construct pHH1_SERA6_loxP (Integ. locus) in clones C4 and D2. The parental 1G5DC clone is included for comparison. A positive control is also included (Ctl). This control consisted of gDNA extracted from a *P. falciparum* clone ('clone 1') which had the pHH1-SERA6chim plasmid integrated into the *SERA6* locus, referred to in [Ruecker et al. 2012]. The pHH1-SERA6chim plasmid used the same endogenous targeting and recodonised region of *SERA6* as the pHH1_SERA6_loxP plasmid used in this study. Primers used for diagnostic PCR to assess integration are shown (half-arrows) (see Table 3.1 for primer sequences). The endogenous locus is expected to produce a product of 1125 bp, the integrant locus is expected to produce a product of 1923 bp.

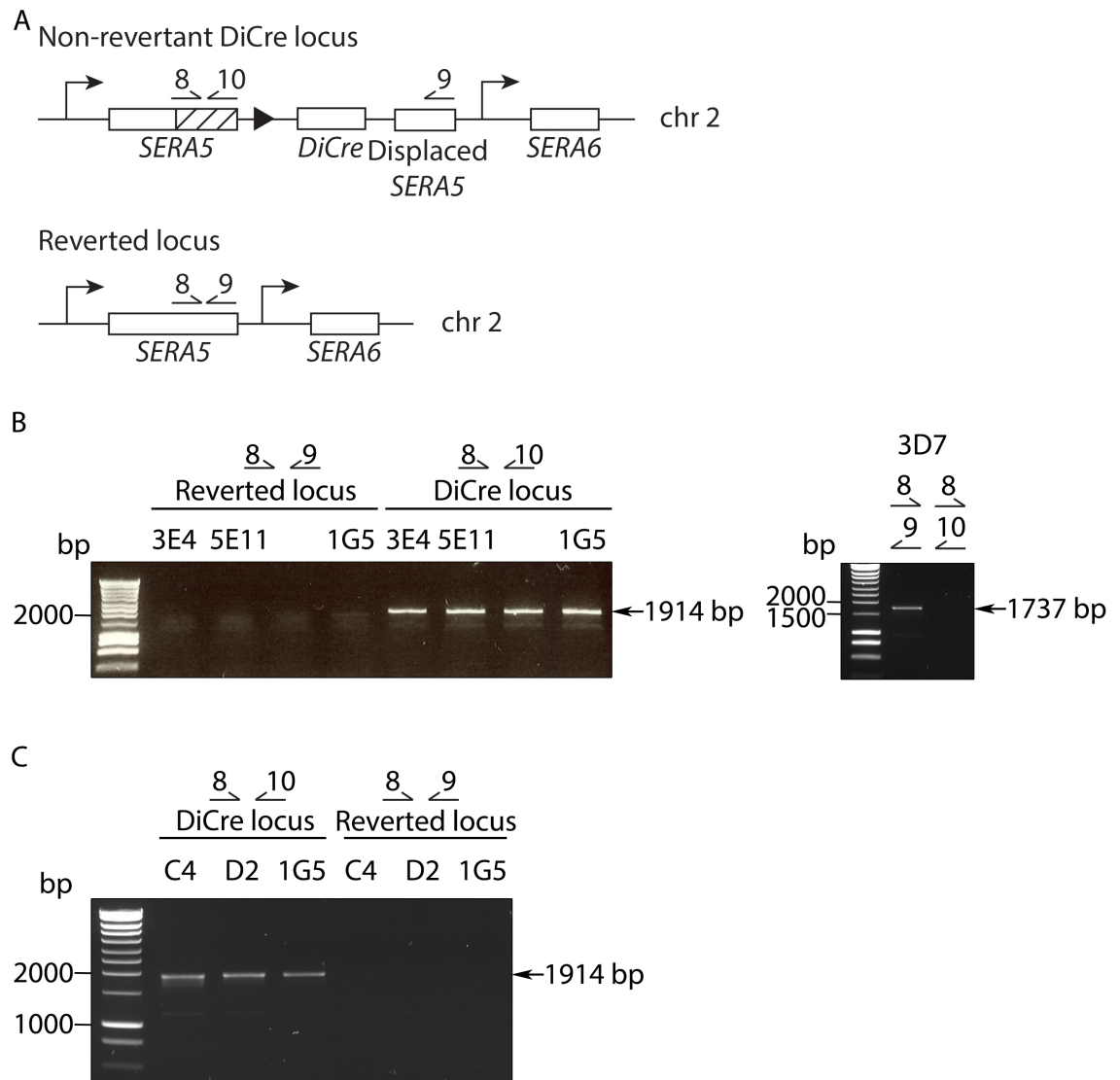


Figure 5.3: The DiCre cassette is present in clones SUB1HA3:loxP clones 3E4 and 5E11 and SERA6:loxP clones C4 and D2. A) Schematic showing architecture of the DiCre locus (chromosome 2) in *P. falciparum* 1G5DC clone (non-revertant DiCre locus), and following reversion of the locus to a wild-type locus (reverted locus). B) Left panel; PCR analysis demonstrating the presence of the DiCre cassette in SUB1HA3:loxP clones 3E4 and 5E11 clones as well as the parental 1G5DC clone. Right panel; PCR analysis showing the lack of DiCre cassette, i.e. wild-type locus, in 3D7 clone *P. falciparum*. C) PCR analysis demonstrating the presence of the DiCre cassette in SERA6:loxP clones C4 and D2 as well as the parental 1G5DC clone. Primers used for diagnostic PCR to assess integration are shown (half-arrows) (see Table 3.1 and [C. R. Collins, S. Das, et al. 2013] for primer sequences). Detection of the non-revertant DiCre locus with primers 8 and 10 was expected to produce a PCR product of 1914 bp. Detection of the reverted, i.e. wild-type locus, with primers 8 and 9 was expected to produce a product of 1737 bp. No product is expected from primers 8 and 9 from the non-revertant locus due to its size (>10 kbp) under the PCR conditions used.

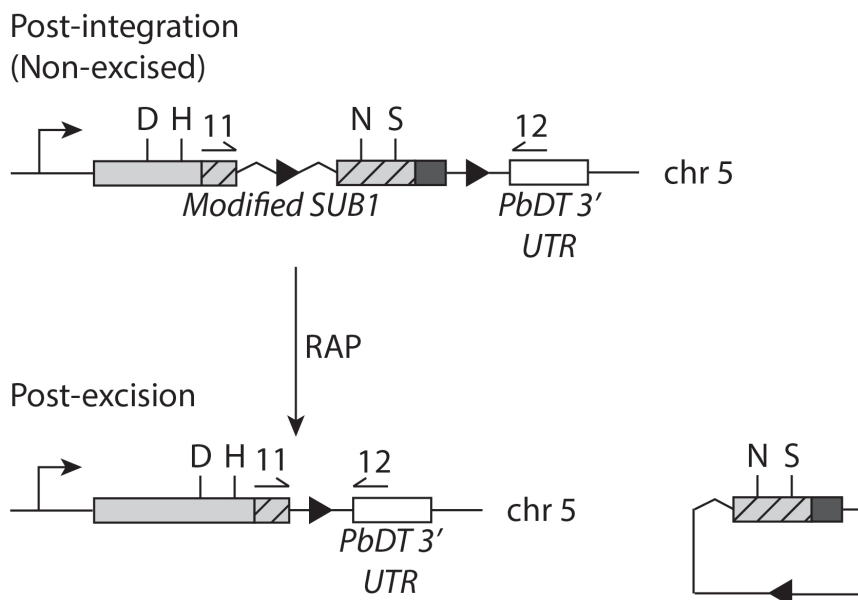


Figure 5.4: Expected *SUB1* locus architecture following RAP-induced excision in *SUB1HA3:loxP* clones 3E4 and 5E11. Schematic showing architecture of the *SUB1* locus (chromosome 5) in *P. falciparum* 1G5DC clone prior to integration of construct pHH1_*SUB1HA3:loxP*, as expected following construct integration, and following DiCre-mediated excision. This construct is designed to integrate by single-crossover homologous recombination and contains an artificial *loxP* site (arrowheads) containing intron (*loxPint*) within a recodonised portion of *SUB1* (hatched) and a *loxP* site downstream of the *SUB1* stop codon such that integration will flox a portion of the gene containing crucial catalytic residues. Following integration, a triple-HA tag (HA3) is fused to the C terminus of *SUB1* (dark grey) and the *PbDT 3'* UTR is placed downstream of *SUB1* to regulate gene expression. Human *DHFR* (*hDHFR*) drug resistance marker is shown. Catalytic residues of *SUB1* are indicated (single letter amino acid code): Asp, D; His, H and Ser S. Oxyanion hole residue is indicated: Asn, N. Primers used for diagnostic PCR to assess excision are shown (half-arrows) (see Table 3.1 for primer sequences). The expected episome deriving from DiCre-mediated excision following RAP-treatment, which truncates the *SUB1* gene and removes the HA3 tag, is shown.

Post-integration
(Non-excised)

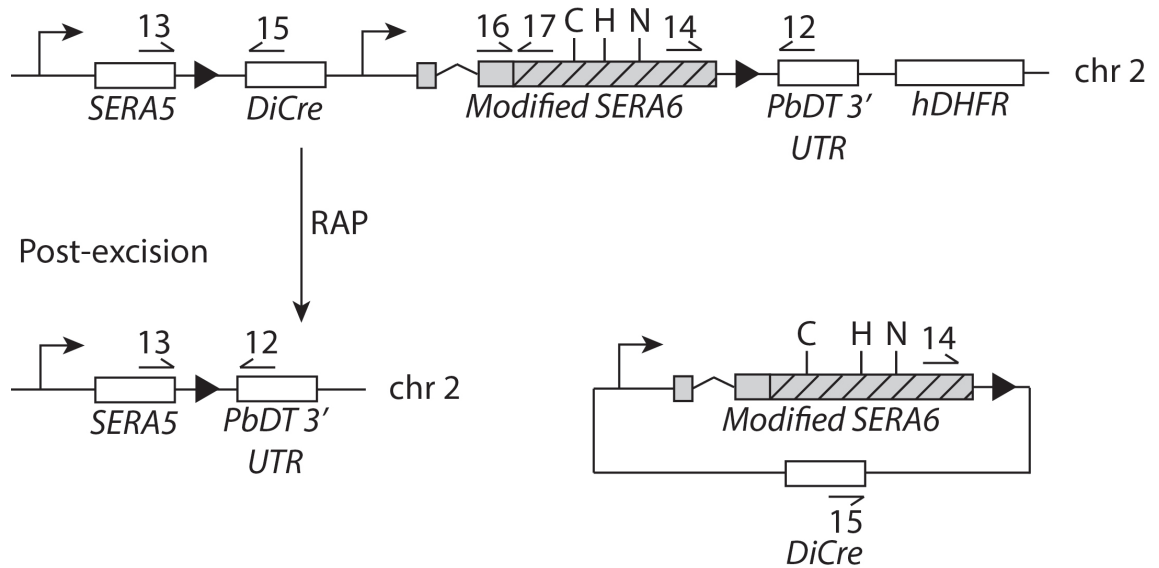


Figure 5.5: **Expected *SERA6* locus architecture following RAP-induced excision in *SERA6:loxP* clones C4 and D2.** Schematic showing architecture of the *SERA6* locus (chromosome 2) in *P. falciparum* 1G5DC clone prior to integration of construct pHH1_*SERA6*_loxP, as expected following construct integration and following DiCre-mediated excision. This construct is designed to integrate by single-crossover homologous recombination and introduces a single *loxP* site (arrowhead) downstream of the *SERA6* stop codon such that integration will flox the entire *SERA6* ORF given the prior existence of a single *loxP* site upstream of *SERA6* in the 1G5DC clone. Following integration the *PbDT* 3' UTR is placed downstream of *SERA6* to regulate gene expression. The upstream *SERA5* gene, human *DHFR* (*hDHFR*) drug resistance marker and DiCre expression cassette are shown. The recodonised portion of *SERA6* is shown (hatched). Predicted conserved catalytic triad residues of *SERA6* are indicated (single letter amino acid code); Cys, C; His, H; Asn, N. Primers used for diagnostic PCR to assess excision are shown (half-arrows) (see Table 3.1 for primer sequences). The expected episome deriving from DiCre-mediated excision following RAP-treatment, which excises the entire *SERA6* ORF and its native promoter is shown.

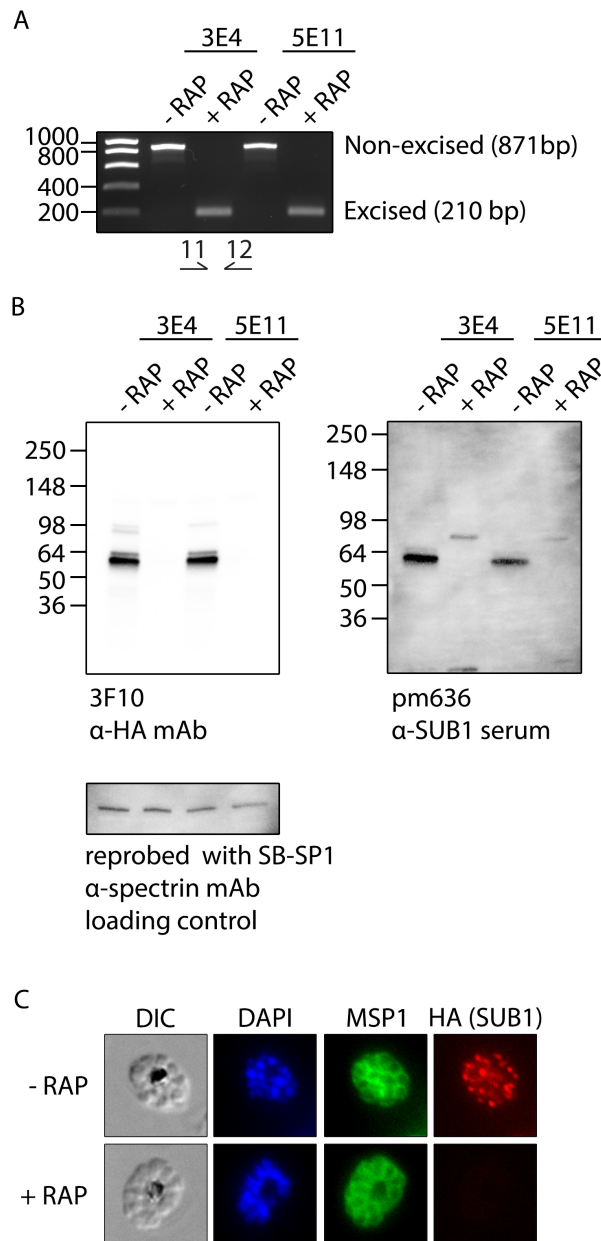


Figure 5.6: RAP-treatment of SUB1HA3:loxP clones 3E4 and 5E11 results in rapid and efficient truncation of the *SUB1* and silencing of expression. A) Diagnostic PCRs performed on gDNA extracted from mature schizonts at the end of cycle 0 (~48h old) confirm that rapid and efficient DiCre-mediated truncation of *SUB1* occurs within a single cycle following RAP-treatment. Primers are indicated (half arrows) (refer to Figure 5.4 and Table 3.1). B) western blots probed with α-HA mAb 3F10 or polyclonal rabbit α-SUB1 demonstrate silencing of *SUB1* expression at the end of cycle 0, following RAP-treatment. The faint ~75 kDa band detected by the rabbit α-SUB1 likely results from expression of the truncated *SUB1* gene. This product likely does not fold properly and therefore does not undergo the co-translational auto-processing event that converts full length 82 kDa SUB1 to the ~47 kDa species (this runs higher than expected on SDS PAGE gels and appears as a ~60 kDa band). As a control a blot was stripped and reprobbed with α-spectrin mAb SB-SP1. C) Representative IFA images demonstrating loss of *SUB1* expression following RAP-treatment. In RAP-treated SUB1HA3:loxP clone 3E4 samples only 0.2% of parasites were scored positive for the presence of the punctate mAb 3F10 α-HA staining (10 positive parasites in 5,056 infected erythrocytes examined). In mock-treated samples 88.2% of parasites scored positive (435 positive parasites in 493 infected erythrocytes examined). Parasites were co-stained with the 89.1 α-MSP1 mAb and DAPI (DNA stain).

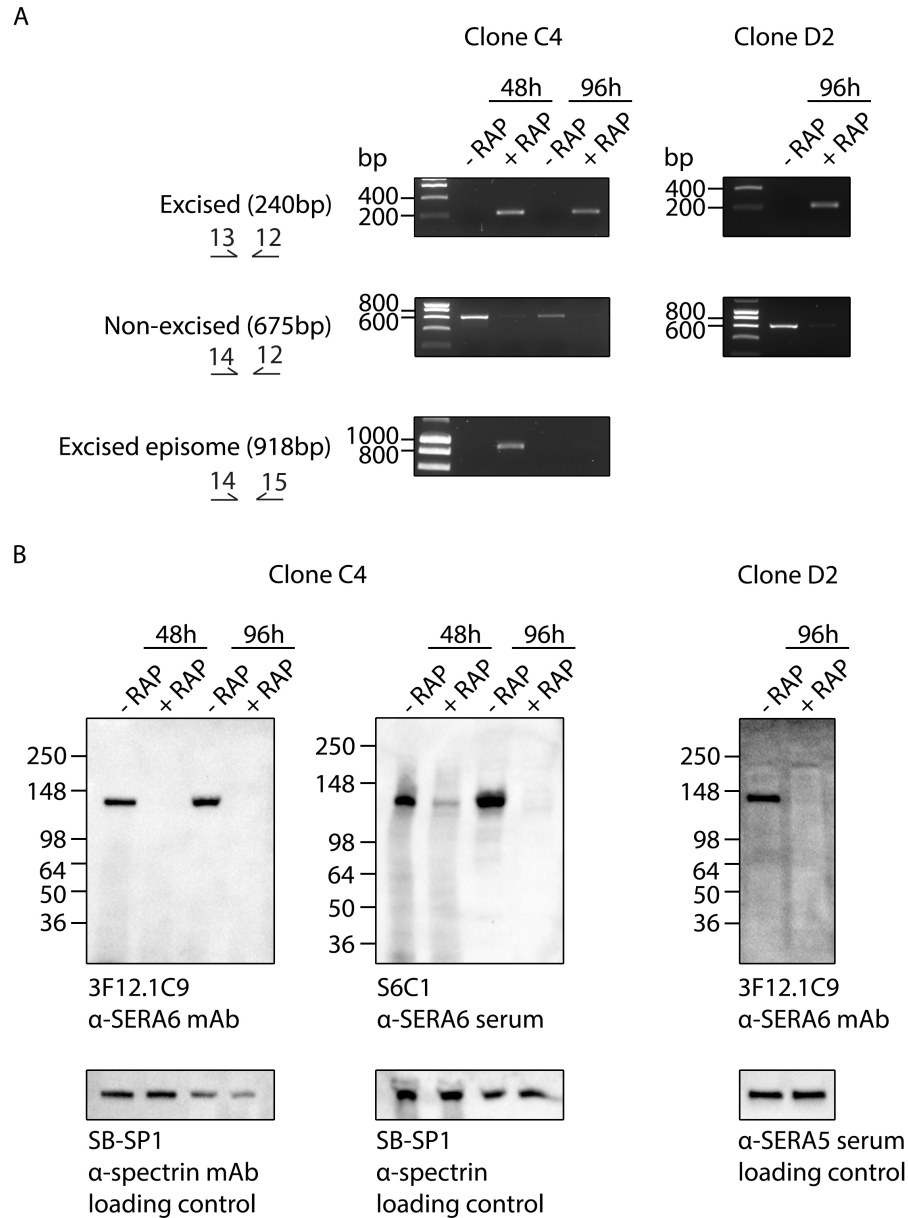


Figure 5.7: RAP-treatment of *SERA6:loxP* clones C4 and D2 results in rapid and efficient excision of *SERA6* and silencing of expression. A) Diagnostic PCRs performed on gDNA extracted from mature schizonts at the end of cycle 0 (~48 h old) confirm rapid and efficient DiCre-mediated excision of *SERA6* following RAP-treatment of *SERA6:loxP* clone C4 parasites. The same pattern was recapitulated by PCR on gDNA samples collected at the end of cycle 1 (~96 h old). PCR on these same gDNA samples demonstrated that the episome which is expected to result from DiCre-mediated excision is detectable at the end of cycle 0 but not at the end of cycle 1. PCRs on clone D2 parasites also demonstrated efficient excision by the end of cycle 1 following RAP-treatment. Primers are indicated (half arrows) (refer to Figure 5.5 and Table 3.1). B) Western blots probed with α-*SERA6* 3F12.1C9 mAb and S6C1 α-*SERA6* rabbit polyclonal serum demonstrate almost complete loss of *SERA6* expression by the end of cycle 0 and no discernible expression by the end of cycle 1 following RAP-treatment. Western blots of protein lysates from clone D2 probed with 3F12.1C9 mAb demonstrated complete loss of protein expression by the end of cycle 1. Loading control blots probed with α-spectrin SB-SP1 mAb or α-*SERA5* polyclonal rabbit serum.

5.2 Silencing of *SUB1* expression ablates proteolytic processing of known SUB1 substrates.

It has been well documented that a number of proteins resident in the PV, as well as on the merozoite surface are proteolytically processed by SUB1 following its discharge from exonemes into the PV lumen [Silmon de Monerri et al. 2011]. These include SERA5, SERA6 and MSP1. In order to confirm that silencing of *SUB1* expression results in the expected loss of SUB1-mediated proteolytic processing of SERA5, SERA6 and MSP1, protein extracts of mock- and RAP-treated SUB1HA3:loxP clone 3E4 parasites were examined for the presence of processed forms of these substrates. Mature mock- and RAP-treated parasites were Percoll purified and incubated in the presence of the reversible PKG inhibitor Compound 2 (C2) for ~4-5h as standard. C2 was then washed off and the parasites rapidly resuspended into Albumax II-free medium in the presence of fresh C2, E64 in the absence of C2 (which arrests egress downstream of C2 arrest and does not inhibit SUB1), or no drug. The parasites were then incubated for a further 30 min before parasite pellets and culture supernatants were collected. SDS-PAGE fractionated protein extracts of all three samples were then examined by western blot. This revealed that following wash-off of C2, either in the presence of E64 or no drug, the expected processing events occurred normally for all substrates in the mock-treated samples (Figure 5.8 and Figure 5.9). This was observed as a decrease in the intensity of each full-length band as well as the appearance of smaller molecular weight bands. Interestingly, in the case of SERA5, it was found that the majority of the processed p50 form was found in the culture supernatant, suggesting that it had 'leaked' from the parasitised cells. In contrast, no, or little evidence of processing could be detected for any of the SUB1 substrates in the RAP-treated samples. This confirmed that following silencing of *SUB1* expression its substrates remained unprocessed. This confirmed that the degree to which expression of *SUB1* was decreased in these clones was sufficient to ablate known SUB1 processes; it also suggested that no redundancy of function exists i.e. these substrates cannot be processed by other proteases. These data confirm that SUB1 process these substrates within the parasite. Previous studies made use of recombinant protein or an inhibitor of SUB1 that was not highly potent, which may not have reflected the reality of the situation in the parasite. Together the data from this study, together with *in vitro* data from previous studies, confirm the role of SUB1 in cleaving these substrates in the parasite.

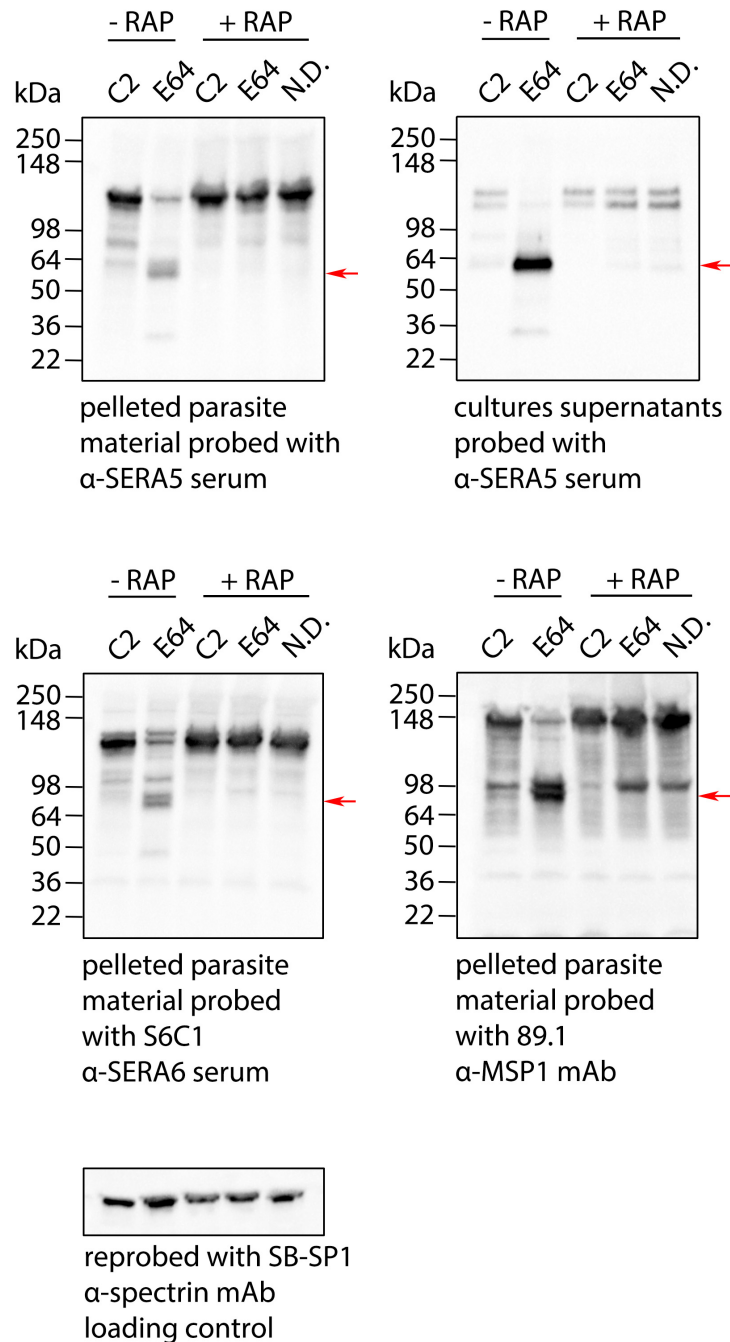


Figure 5.8: **Silencing of *SUB1* expression results in loss of processing of the *SUB1* substrates SERA5, SERA6 and MSP1.** Western blots of pelleted parasite material and culture supernatants from mature Percoll-purified schizonts probed with α-SERA5 rabbit polyclonal serum, α-SERA6 rabbit polyclonal serum S6C1 and the α-MSP1 mAb 89.1. C2-arrested schizonts at the end of cycle 0 following mock-treatment (- RAP) or RAP-treatment (+ RAP) were washed into Albumax II-free medium containing fresh C2, E64 in the absence of C2 (E64), or no drugs (n.d.). Cleaved forms that result from *SUB1* processing in mock-treated cultures can be seen and are indicated by arrows. Processed forms are not readily detectable in RAP-treated samples. The processed p50 form of SERA5 had leaked from the parasitised cells into the culture supernatant following reversal of C2 arrest in the absence of E64 or no drug. A control blot was probed with the α-spectrin mAb SB-SP1.

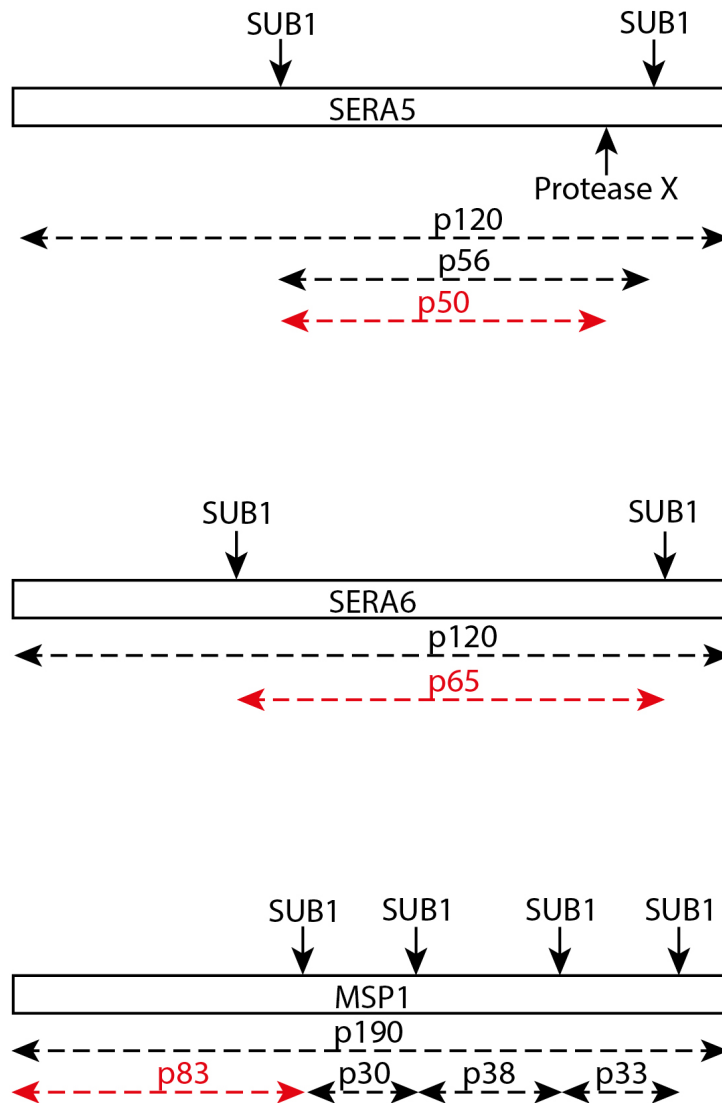


Figure 5.9: **SUB1 processing of SERA5, SERA6 and MSP1.** A schematic showing the processing SUB1 mediated events of SERA5 [Stallmach et al. 2015], SERA6 [Ruecker et al. 2012] and MSP1[S. Das et al. 2015] and the major products that result, as well as the 'protease X' mediated cleavage that occurs to SERA5. Intermediate products are not represented. The products shown in red correspond to the major observable products detected by western blot in Figure 5.8 and indicated with red arrows.

5.3 *SUB1* and *SERA6* are essential for the viability of the asexual blood stage cycle of *P. falciparum* in vitro.

5.3.1 Conditional silencing of *SUB1* or *SERA6* results in a severely diminished replication rate.

In order to assess whether *SUB1* and *SERA6* are essential for the viability of asexual blood stage cycle parasites, standard flow-cytometry based replication assays were performed on mock- and RAP-treated cultures of SUB1HA3:loxP clones 3E4 and 5E11 as well as SERA6:loxP clones C4 and D2. In the case of clones 3E4 and 5E11, cultures were diluted to the desired densities during cycle 0, the same cycle in which they were mock/RAP-treated. It was previously discovered that RAP-treated SERA6:loxP clone C4 parasites proliferated at the end of cycle 0 as efficiently as those that were mock-treated; parasitaemia counts (by examination of Giemsa stained thin films) of mock- and RAP-treated cultures during cycle 1 were 10.6% and 10.4% respectively. As such clone C4 and clone D2 cultures were established during cycle 1 for the purposes of the replication assay. Parasitaemias were measured at the onset of the assay and over the ensuing two cycles by flow cytometry. The results demonstrated dramatic reductions in parasite replication rate resulting from RAP-mediated silencing of *SUB1* or *SERA6* expression (Figure 5.10).

The flow-cytometry based replication assay only assesses parasite replication over a relatively short period. In order to assay viability over a longer period of time, mock- and RAP-treated cultures of SUB1HA3:loxP clone 3E4 and SERA6:loxP clone C4 were passaged for up to 20 days, such that parasitaemia never exceeded 5%. To evaluate the fate of the excised parasites and the initially small fraction of co-existing non-excised parasites, gDNA was periodically extracted from both mock- and RAP-treated cultures in parallel to assess the prevalence of excised and non-excised loci by PCR. This revealed that in both cases the initially minor populations of non-excised parasites gradually out-grew the excised (Δ *SUB1* or Δ *SERA6*) parasites, which were undetectable by PCR by the end of the assay (Figure 5.11). This qualitative approach confirmed the results of the short-term replication assay in showing that disruption of the *SUB1* or *SERA6* genes results in severe growth defects. Furthermore, it suggested that even over extended periods Δ *SUB1* and Δ *SERA6* parasites are unable to replicate productively. PCR product corresponding to excised parasites persisted following RAP-treatment of both SUB1HA3:loxP clone 3E4 and SERA6:loxP clone C4 for up to 8 and 4 cycles respectively. This likely results from excised DNA of dead parasites present in the culture supernatant. This is because the precipitous decline in parasitaemia in the RAP-treated cultures meant the cultures could not be passaged for some time. In contrast, in mock-treated cultures parasitaemia increased exponentially as normal and cultures had to be passaged to avoid parasite death due to nutrient starvation and toxic buildup. The excised DNA of dead parasites in the RAP-treated culture likely remains in solution which can be detected by PCR. Eventually the outgrowth of the initially minor population of non-excised parasites in the RAP-treated culture allowed the passage of these cultures which would dilute this DNA such that PCR product was no longer readily amplified from it.

5.3.2 Examination of parasite growth by plaque assay demonstrates that disruption of *SUB1* or *SERA6* abolishes parasite replication.

The flow-cytometry based replication assays presented above assess parasite replication rate at the population level for only a few erythrocytic growth cycles. The use of diagnostic PCR to assess the relative prevalence of non-excised and excised parasites was an attempt to examine parasite growth over a longer period of time and to examine the genotypes present in the population, given that DiCre mediated gene excision is never complete and results in a genetically heterogeneous population containing some non-excised, effectively wild-type, parasites. This still only examines the population level however and is by no means quantitative. To assess the impact of gene disruption on parasite viability at the clonal level, parasite growth was examined using the plaque assay presented in Chapter 4. This method assays the ability for individual parasite clones that are dispensed into a microplate well to undergo successive rounds of invasion, replication and egress. The readout of the plaque assay is the presence or absence of individual plaques which result from single parasites. Combined with the fact that it is performed at close to limiting dilution, with parasitaemias never exceeding a level where they need passaging, the ability for the original initiating parasite clones to proliferate is examined, in effect, in isolation from other parasites. The plaque assay therefore overcomes the ambiguity which can result from the previously presented methods that provide a readout at the population level. To this end, cultures were diluted equivalently to densities of between 1-50 parasites per well following mock- and RAP-treatment and dispensed into 96-well flat-bottomed microplates. Plaques were quantified 14 days later. For each individual experiment the number of plaques forming in wells containing RAP-treated culture was calculated as a percentage of those forming in an equivalent number of wells containing mock-treated cultures: this ratio was termed the relative plaque forming ability (RPFA). Dramatic reductions in RPFA were seen as a result of RAP-mediated disruption of either *SUB1* or *SERA6*. In the case of SUB1HA3:loxP clone 3E4 the average RPFA of RAP-treated cultures was 2.6% (mean = 2.6%, SD = 1.4, n = 2) (Figure 5.12). In other words, for every 100 plaques formed in wells containing mock-treated culture only 2.6 plaques formed in an equivalent number of wells containing RAP-treated culture. The same experiment performed on clone 5E11 revealed a similar effect, generating a RPFA value of 1.4% (mean = 1.4%, SD = 0.4, n = 2) (Figure 5.12). A number of wells containing single plaques produced by RAP-treated clone 3E4, were expanded. gDNA was extracted from these cultures and analysed by PCR. In all cases, evidence of the non-excised locus was detected by PCR (Figure 5.13). The significant reductions in RPFA following RAP-treatment, along with this demonstration that only viable non-excised, i.e. effectively wild-type parasites, could be isolated, provides further evidence that Δ *SUB1* parasites are not viable in *in vitro* culture. Similar results were obtained upon analysis of the Δ *SERA6* phenotype. The RPFA of RAP-treated *SERA6*:loxP clones C4 and D2 was found to be 0.6% (mean = 0.6, SD = 0.7, n = 2) and 0.8% (mean = 0.8, SD = 0.4, n = 2) respectively (Figure 5.12). Similarly, from a single experiment performed on clone C4, a single well contained a single plaque following-RAP treatment. This culture was expanded and gDNA extracted and analysed by PCR. This revealed this parasite clone to be non-excised (Figure 5.13). Similarly therefore, dramatic reductions in RPFA were seen following RAP-induced silencing of *SERA6* expression and

only non-excised parasites could be isolated. Together, the data from these various assays convincingly demonstrates that *SUB1* and *SERA6* are both essential for viability of the asexual blood stage of *P. falciparum* in *in vitro* culture.

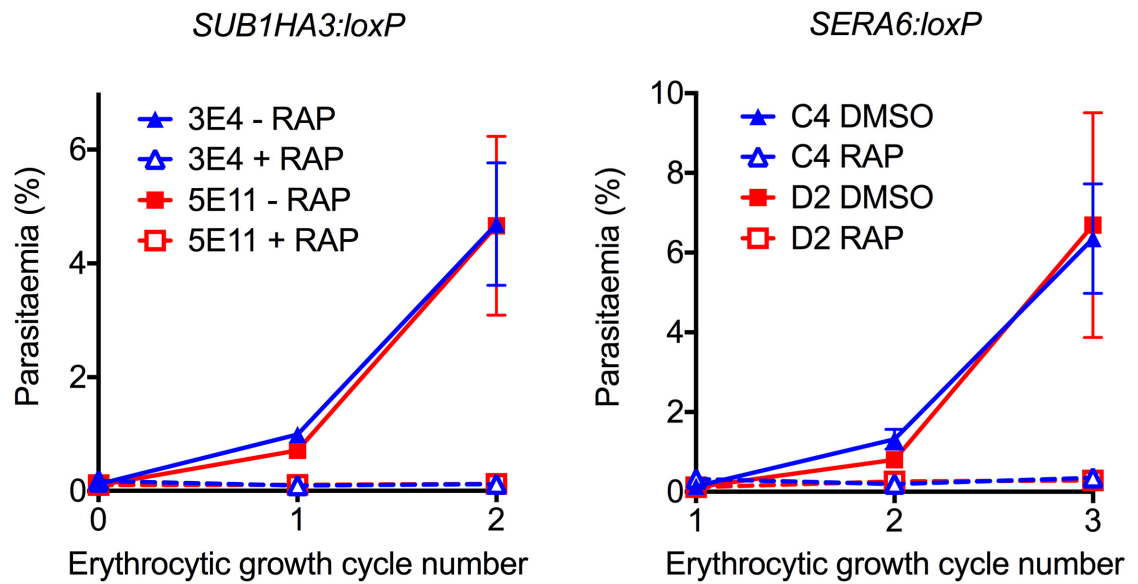


Figure 5.10: **Silencing of *SUB1* or *SERA6* expression results in dramatic reductions in parasite replication rates.** Plots showing growth curves (parasitaemias counted by flow-cytometry) of *SUB1HA3:loxP* clones 3E4 and 5E11 and *SERA6:loxP* clones C4 and D2 following mock-treatment (-RAP) or RAP-treatment (+ RAP) of synchronous ring-stage cultures. Cultures of clones 3E4 and 5E11 were established ~24 h post treatment (cycle 0) and cultures of clones C4 and D2 were established ~72 h post treatment (cycle 1) to account for the delayed phenotype resulting from *SERA6* disruption. Solid lines represent mock-treated samples, dashed lines represent RAP-treated samples error bars indicate mean \pm 1 standard deviation from two replicate experiments performed using blood from different donors.

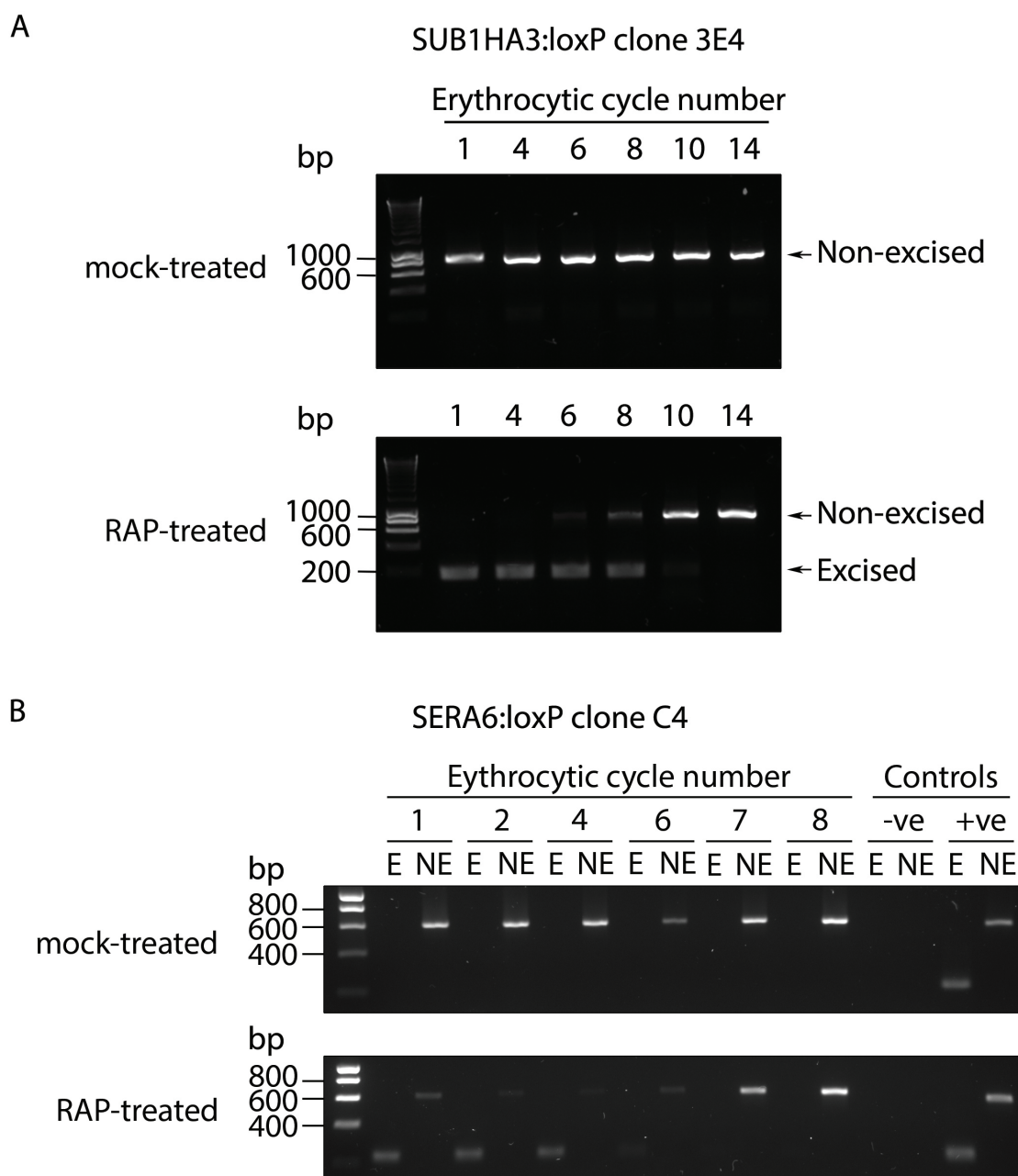


Figure 5.11: PCR analysis of passaged RAP-treated cultures demonstrates that Δ SUB1 and Δ SERA6 parasites cannot proliferate. A) Diagnostic PCR analysis of parasite gDNA extracted from mock- or RAP-treated cultures of SUB1HA3:loxP clone 3E4. Signals corresponding to PCR amplicons demonstrating the presence of the excised *SUB1* locus become less intense with time, as PCR amplicons demonstrating presence of non-excised locus appear and become more intense. Primers 1 and 2 were used for the detection of excised and non-excised locus (refer to Figure 5.4, Figure 5.6 and Table 3.1). B) Diagnostic PCR analysis of parasite gDNA extracted from mock- or RAP-treated cultures of SERA6:loxP clone C4. Signals corresponding to PCR amplicons demonstrating the presence of the excised *SERA6* locus become less intense with time, as PCR amplicons demonstrating presence of non-excised locus appear and become more intense. Separate primer pairs are used to examine for non-excised and excised *SERA6* loci: Excised (E) primers 3 and 2; Non-excised (NE) primers 4 and 2 (refer to Figure 5.5, Figure 5.7 and Table 3.1).

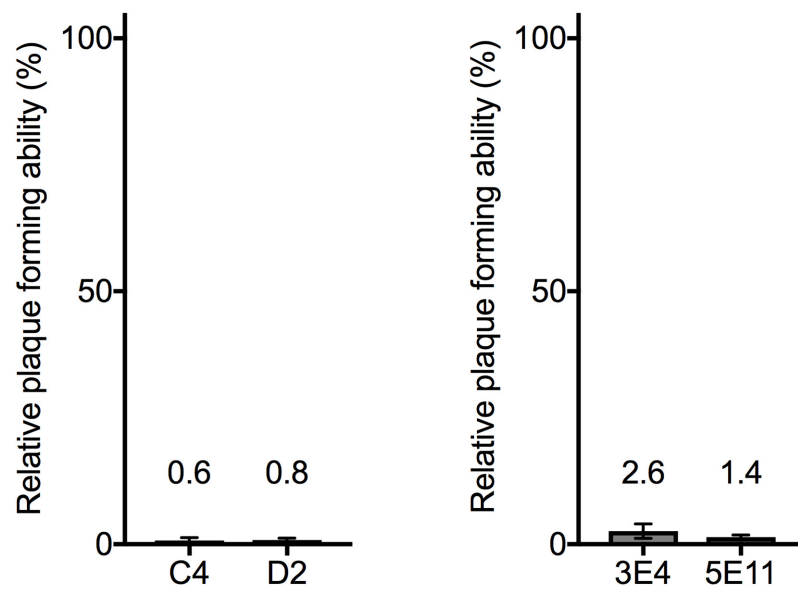


Figure 5.12: **Silencing of *SUB1* or *SERA6* results in dramatic reductions in relative plaque forming ability.** Plots showing RPFAs of SUB1HA3:loxP clones 3E4 and 5E11 and SERA6:loxP clones C4 and D2 following RAP-treatment. Cultures were mock- or RAP-treated and dispensed into microplates at equivalent parasite densities. Plaques were enumerated after 14 days of incubation and the RPFA of RAP-treated cultures was calculated as the number of plaques forming in wells containing RAP-treated cultures as a percentage of those formed in wells containing mock-treated cultures. Error bars show ± 1 standard deviation from two replicate experiments using blood from different donors.

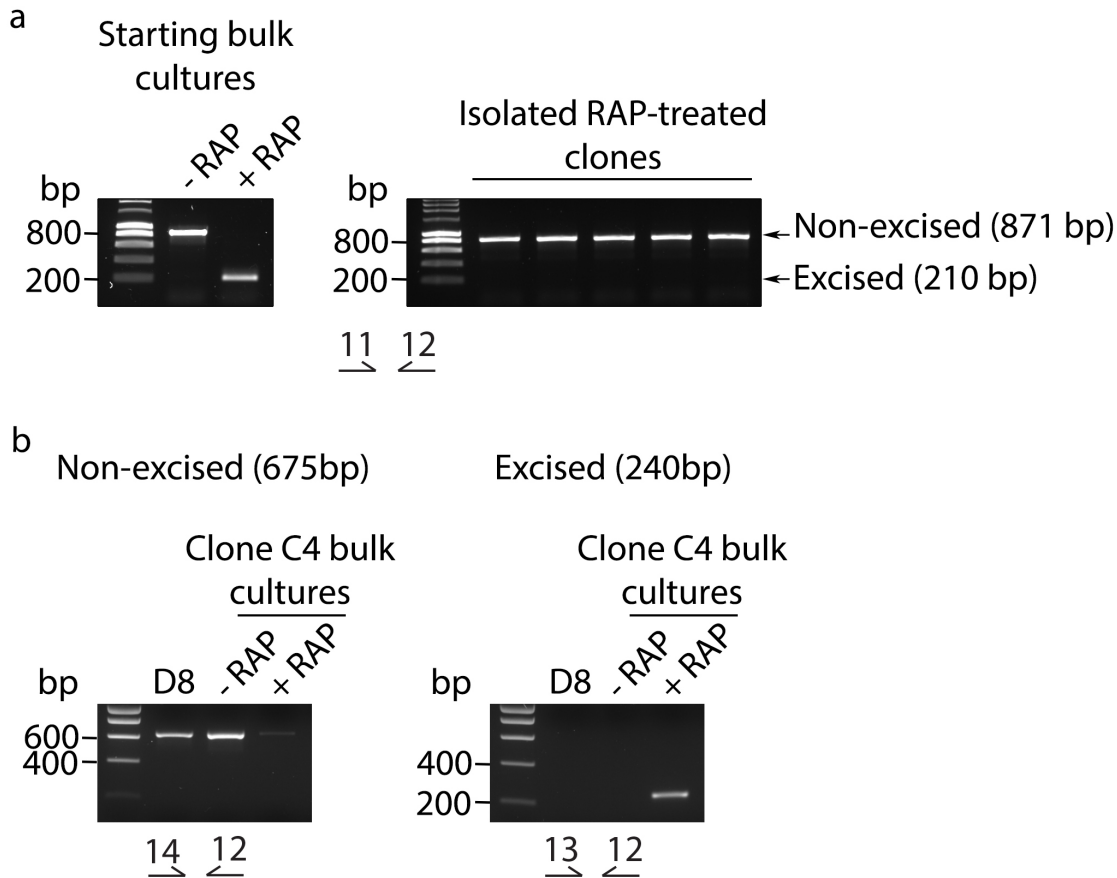


Figure 5.13: Genotyping of clones isolated following RAP-treatment of SUB1HA3:loxP clone 3E4 and SERA6:loxP clone C4 reveals them to be non-excised. A) Diagnostic PCR analysis examining the presence of excised or non-excised loci of starting bulk cultures of mock- (- RAP) or RAP-treated (+ RAP) SUB1HA3:loxP clone 3E4 prior to dispensing in microplate wells (left) and clones isolated from wells containing RAP-treated culture following plaque assay (right). A single PCR detects both excised and non-excised loci, primers used are indicated (half arrows) (refer to Figure 5.4, Figure 5.6 and Table 3.1). No isolated RAP-treated clones were found to have undergone excision corroborating the conclusion that *SUB1* is essential and knockouts are not viable. B) Diagnostic PCR analysis examining the presence of excised or non-excised locus of starting bulk cultures of mock- (- RAP) or RAP-treated (+ RAP) SERA6:loxP clone C4 prior to dispensing in microplates (left) and a single clone (called D8) isolated from a well containing RAP-treated culture (right). Primers used are indicated (refer to Figure 5.5, Figure 5.7 and Table 3.1). No isolated RAP-treated clones were excised corroborating the conclusion that SERA6 is essential and knockouts cannot be isolated.

5.4 Transgenic expression of wild-type copies of *SUB1* or *SERA6* is able to complement the respective growth defects resulting from disruption of the chromosomal copies of these genes.

To confirm that loss of viability of $\Delta SUB1$ and $\Delta SERA6$ parasites results specifically from silencing of these genes and not from unintended chromosomal alterations associated with Cre recombinase activity, rescue of the growth defects was attempted by genetic complementation. To do this, episomal constructs were designed for transgenic expression of wild-type *SUB1* in SUB1HA3:loxP clone 3E4 and WT *SERA6* in SERA6:loxP clone C4. Clones 3E4 and C4 were also transfected with identical constructs lacking the expression cassettes for *SUB1* or *SERA6*, referred to as 'empty vectors', as controls. Following transfection of the episomal constructs and drug selection with blasticidin-S, disruption of the chromosomal genes was induced by RAP-treatment and growth of the lines was assessed by plaque assay. This demonstrated that a significant increase in RPFA was achieved in RAP-treated lines harbouring the episomal wild-type genes, compared to RAP-treated lines harbouring empty vectors. In the case of SUB1HA3:loxP clone 3E4 harbouring the episomal WT *SUB1* vector, the relative plaque forming ability was 13.5 (mean = 13.5, SD = 1.0, n = 2) compared to an RPFA of only 2.8 (mean = 2.8, SD = 1.7, n = 2) for those parasites harbouring the empty vector (Figure 5.14). An unpaired two-sample t-test confirmed this difference to be significant ($t = 7.702$, d.f. = 2, $p = 0.0164$). For clone C4 parasites harbouring the episomal WT *SERA6* gene the figure was 88.1% (mean = 88.1, SD = 6.3, n = 2) compared to an RPFA of only 0.4% (mean = 0.4, SD = 0.5, n = 2) for those harbouring the empty control vector (Figure 5.15). An unpaired two-sample t-test confirmed this difference to be significant ($t = 19.65$, d.f. = 2, $p = 0.0026$). In a similar approach to that outlined in Section 5.3.2, for both clones, those wells which contained RAP-treated culture and in which plaques were observed, were expanded and parasite gDNA examined by PCR to assess the genotypes of these lines (they were not necessarily clonal). It was found that in every case examined, parasite lines had undergone gene excision as expected (Figure 5.16). For SUB1HA3:loxP clone 3E4 the complementing episomal construct was detected by PCR using the same primers (Figure 5.16).

The isolation of parasite lines which had excised the chromosomal *SUB1* or *SERA6* genes but that possessed episomal gene copies acted as further evidence that expression of these genes from episomal constructs was sufficient to rescue the otherwise lethal defect resulting from chromosomal gene silencing. The complementation data confirmed that the loss of viability seen from silencing of these genes is indeed due to loss of *SUB1* or *SERA6* expression and not unintended chromosomal changes that might occur upon activation of Cre recombinase activity.

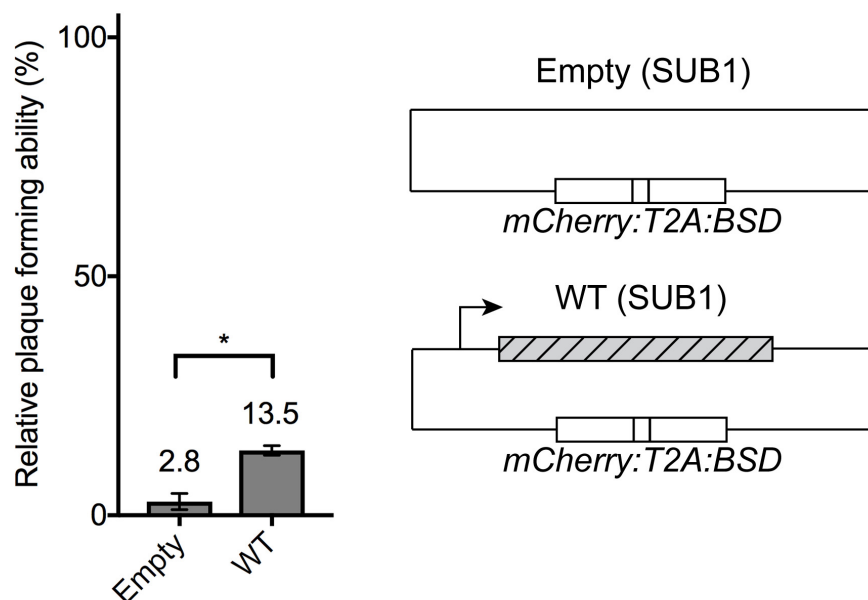


Figure 5.14: **Genetic complementation confirms *SUB1* essentiality.** SUB1HA3:loxP clone 3E4 harbouring the WT *SUB1* complementation construct (WT SUB1) displayed a significant increase in RPFA following RAP-treatment (mean = 2.8%, SD = 1.7, n = 2) compared to clone 3E4 harbouring an empty control vector (Empty) (t=7.702, d.f.=2, p=0.0164). Statistical significance was determined by unpaired two-tailed t-test: Empty vs WT (mean = 13.5%, SD = 1.0, n = 2). Error bars show ± 1 standard deviation. Bars show means of two repetitions in blood from different donors. Schematics of complementation constructs are shown. The recodonised SUB1 gene is shown shaded grey/hashed, under control of its native promoter.

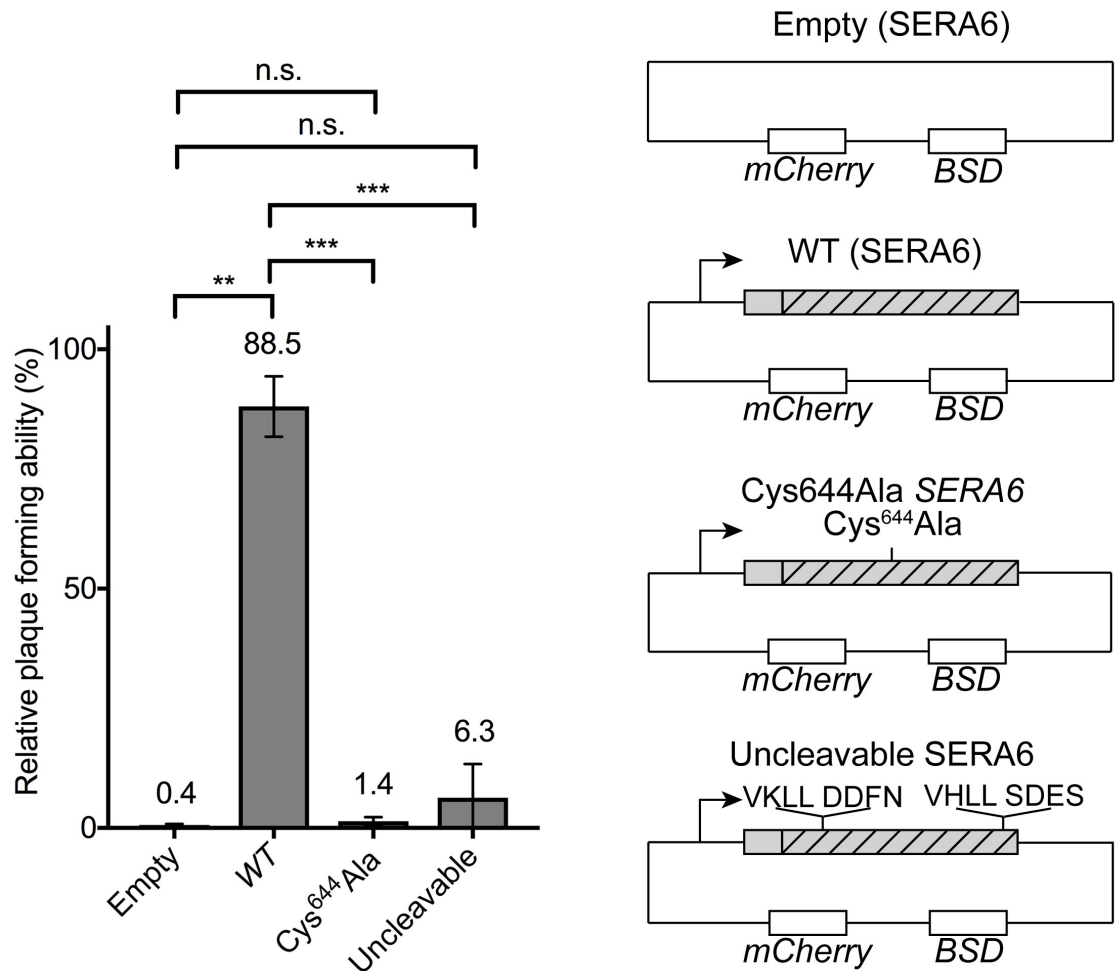
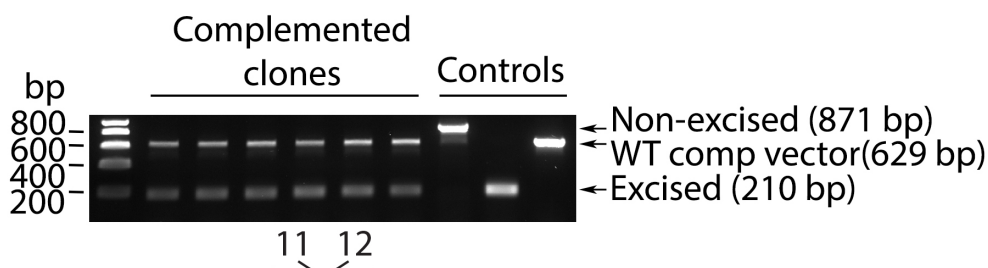


Figure 5.15: Genetic complementation confirms *SERA6* essentiality, and an indispensable enzymatic role for *SERA6*, as well as the requirement for SUB1-mediated processing to activate *SERA6* function. *SERA6*:loxP clone C4 parasites harbouring the wild-type *SERA6* complementation construct (WT *SERA6*) displayed a significant increase in RPFA following RAP-treatment (mean = 88.1%, SD = 6.3, n = 2) compared to clone C4 harbouring an empty control vector (Empty(*SERA6*)) (mean = 0.4%, SD = 0.5, n = 2). The same clone harbouring a construct designed for episomal expression of a mutant form of *SERA6*, possessing a Cys⁶⁴⁴Ala substitution, which is expected to ablate enzymatic activity, did not significantly restore the RPFA of parasites following RAP-treatment (mean = 1.4%, SD = 0.9, n = 4). Similarly, a construct with an ‘uncleavable’ mutant of *SERA6* that cannot be proteolytically processed by SUB1, by virtue of di-leucine substitutions in the P1 and P2 positions of the cleavage sites, fails to restore the RPFA of parasites (mean = 6.3%, SD = 7, n = 4). Error bars show ± 1 standard deviation. Bars show means of between two and four repetitions in blood from different donors. Statistical significance was determined by two-tailed unpaired t-tests: Empty vs WT *SERA6* (t = 19.65, d.f. = 2, p = 0.0026); WT vs Cys⁶⁴⁴Ala (t = 30.96, d.f. = 4, p < 0.0001); WT vs Uncleavable (t = 13.8, d.f. = 4, p = 0.0002); Empty vs Cys⁶⁴⁴Ala (t = 1.557, d.f. = 4, p = 0.1944); Empty vs Uncleavable (t = 1.136, d.f. = 4, p = 0.3195). Significance levels indicated: p < 0.001, ***; p \leq 0.01, **; p \leq 0.05, *. Schematics of complementation constructs are shown. The *SERA6* gene is shown shaded grey (recodonised portions are hashed) under control of its native promoter.

A *SUB1HA3:loxP* clone 3E4 transfected with WT *SUB1*



B *SERA6:loxP* clone C4 transfected with WT *SERA6*

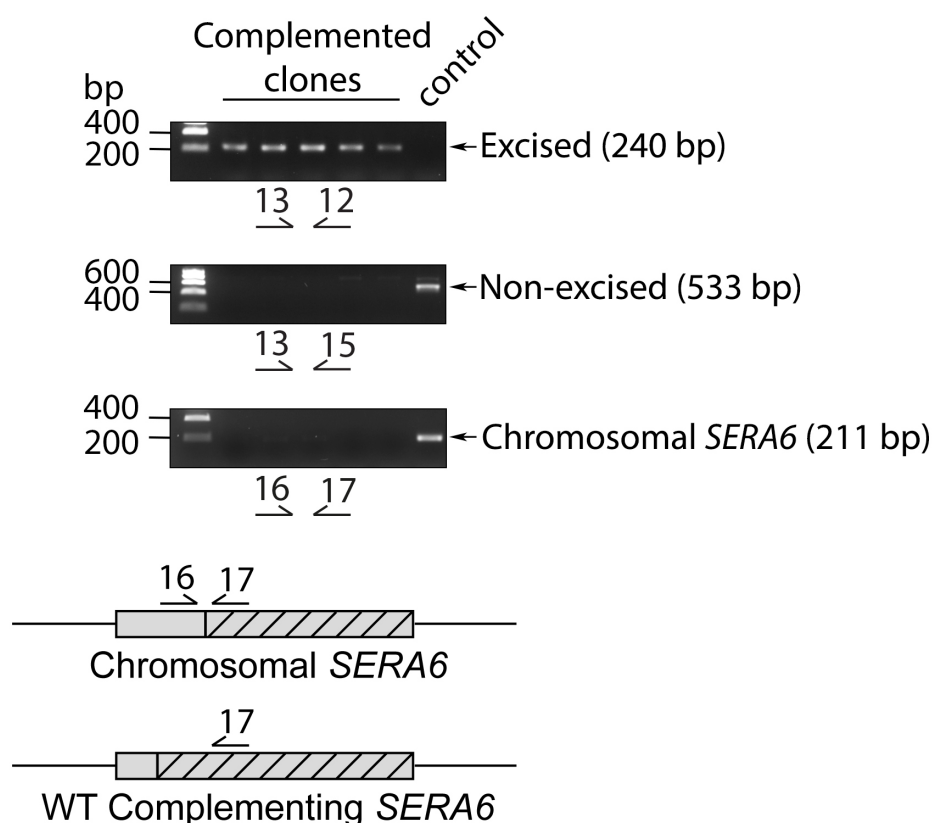


Figure 5.16: **Genotyping of clones obtained following RAP-treatment confirms genetic complementation.** A) Diagnostic PCR analysis examining the presence of excised or non-excised loci of clones isolated from wells containing RAP-treated *SUB1HA3:loxP* clone 3E4 harbouring the WT *SUB1* complementation vector. Primers used are indicated (refer to Figure 5.4, Figure 5.6 and Table 3.1). B) Diagnostic PCR analysis examining the presence of excised or non-excised locus of clones isolated from wells containing RAP-treated *SERA6:loxP* clone C4 harbouring the WT *SERA6* complementation vector. In addition, a third PCR strategy was employed which is specific for the chromosomal *SERA6* gene, but not the complementing copy (which has a more extensive recodonised portion). This confirmed that the chromosomal copy of *SERA6* is not present. A schematic is shown indicating difference in size of recodonised portions of the chromosomal *SERA6* and the episomal WT complementing *SERA6* (bottom). Primers used are indicated (refer to Figure 5.5, Figure 5.7 and Table 3.1). In both A) and B) all isolated clones were excised at the chromosomal loci.

5.5 Failure to genetically complement Δ *SERA6* parasites with mutant forms of *SERA6* demonstrates that *SERA6* plays an enzymatic role and that processing by SUB1 is required for function.

Having established genetic complementation of Δ *SERA6* parasites with an episomal copy of WT *SERA6*, gene function was further interrogated by complementation with mutant forms of *SERA6*. Previous work from the laboratory of Michael Blackman [Ruecker et al. 2012] has indicated that *SERA6* Cys⁶⁴⁴ corresponds to the canonical active site nucleophilic Cys residue required for catalytic activity of all papain-like cysteine proteases. The same study produced evidence that SUB1-mediated processing of *SERA6* is important for parasite viability. To build on these data using the complementation system, a mutant form of *SERA6* which contained a Cys⁶⁴⁴Ala substitution (expected to ablate proteolytic activity) as well as a form in which the SUB1 processing sites had been disrupted by di-leucine substitutions in the P1 and P2 positions of the two cleavage sites (shown to disrupt SUB1 cleavage [Yeoh et al. 2007]), were transfected into *SERA6*:loxP clone C4. Plaque assays revealed that the RPFA of RAP-treated parasites harbouring the episomal Cys⁶⁴⁴Ala mutant (mean = 1.4, SD = 0.9, n = 4) did not differ significantly from that of similarly RAP-treated parasites harbouring the empty control vector (Figure 5.15) as determined by two-tailed unpaired t-test (t = 1.557, d.f. = 4, p = 0.1944). This finding that the Cys⁶⁴⁴Ala mutant cannot reverse the lethal growth defect resulting from disruption of the chromosomal gene strongly suggests that *SERA6* plays an essential enzymatic role in maintaining viability of asexual blood stage parasites. In the case of parasites harbouring the episomal uncleavable mutant of *SERA6* the RPFA of RAP-treated parasites was also found to be not significantly different from that of RAP-treated parasites harbouring the empty control vector (mean = 6.3, SD = 7.0, n = 4) as determined by two-tailed unpaired t-test (t = 1.136, d.f. = 4, p = 0.3195) (Figure 5.15). This is in accord with the model that processing of *SERA6* by SUB1 is required for *SERA6* function. These data are consistent with *SERA6* playing a proteolytic role in the parasite, and being expressed as a zymogen form which requires processing to activate this function.

5.6 SUB1 is required for rupture of the PVM and for all of the morphological changes that occur downstream of PKG activation.

Having established that *SUB1* is clearly essential for parasite viability, the effect of *SUB1* disruption specifically on egress was then assessed. Live time-lapse microscopy was used to examine egress of mock- and RAP-treated *SUB1*HA3:loxP clone 3E4 parasites at the end of the cycle in which they were treated (cycle 0). For this, mature Percoll enriched schizonts were incubated in the presence of C2 for ~4.5-6 h, then washed, followed by immediate examination by time-lapse DIC microscopy. The Δ *SUB1* parasites were clearly deficient in egress with no release of merozoites seen. Of particular interest, the PVM

apparently did not rupture in $\Delta SUB1$ parasites as determined by the lack of discernible merozoites within the erythrocyte in the DIC imaging, (Figure 5.17) and rounding up was also not observed. In fact, the phenotype exhibited was indiscernible from that produced by continued inhibition of PKG by C2 (not shown). Conversely, in mock-treated parasites there comes a point where merozoites suddenly become discernible at the same time that rounding-up of the parasitised erythrocyte occurs prior to erythrocyte membrane rupture and merozoite release (Figure 5.17). To quantify the defect, the percentage of parasites in which PVM rupture was observed in mock- and RAP-treated cultures was scored for. PVM rupture deemed to have occurred when individual merozoites became readily discernible. The raw percentage of PVM rupture events for each video was normalised, with the highest percentage of PVM rupture from any single video within a single experiment (invariably a mock-treated sample) being treated as 100%. This was to control for the differences in parasite maturity, and therefore egress rate, between replicate experiments conducted on different days, using different parasite samples. As shown in Figure 5.17, normalised PVM rupture was significantly higher in mock-treated cultures (mean = 90.8%, SD = 12.72, n = 5) than in RAP-treated cultures (mean = 4.1%, SD = 6.7, n = 5). An unpaired two-sample t-test revealed this difference to be highly significant ($t = 13.49$, d.f. = 8, $p < 0.0001$). Similar time-lapse examination of a clone derived from RAP-treatment of SUB1HA3:loxP clone 3E4 harbouring the WT *SUB1* complementation construct which was confirmed to be excised at the chromosomal *SUB1* locus (see Section 5.4) revealed that normal PVM rupture and egress had been restored to these parasites (Figure 5.18), confirming the role of SUB1 as an effector of these morphological changes.

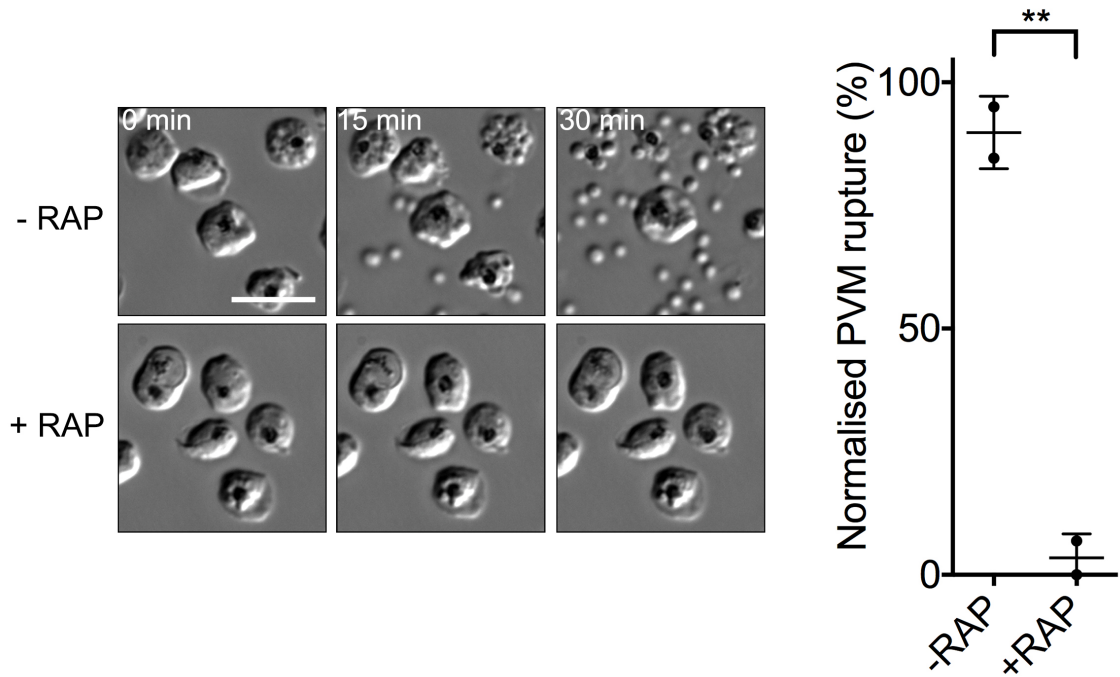


Figure 5.17: **Silencing of *SUB1* expression ablates all the morphological changes that follow PKG activation.** Left-hand side, DIC images from time-lapse microscopy of mature SUB1HA3:loxP clone 3E4 schizonts. Parasites were mock- (- RAP) or RAP-treated (+ RAP) at ring-stage then Percoll purified at the end of cycle 0 and C2 arrested. C2 arrest was reversed by washing of parasites to allow egress to proceed with parasites immediately examined microscopically for 30 min. Time from commencement of microscopy is indicated. Scale bar, 10 μ m. In mock-treated parasites merozoite release is clearly discernible, in contrast to RAP-treated parasites which undergo none of the morphological changes that are usually visible in time-lapse analysis, including rounding up, PVM rupture which allows increased visibility and movement of merozoites, and final rupture of the erythrocyte membrane to allow merozoite release. Right-hand side, quantification revealed that significantly fewer RAP-treated parasites underwent PVM rupture (mean = 4.1, SD = 6.7, n = 5) compared to mock-treated parasites (mean = 90.8, SD = 12.7, n = 5). Data were collected from 5 videos each of mock- and RAP-treated parasites from 2 replicate experiments (total number of observed PVM rupture events in mock-treated parasites, 226). Percentage PVM rupture of each replicate within a single experiment was normalised with the replicate that demonstrated the greatest percentage egress in a single experiment treated as 100%. Statistical significance was determined by two-tailed unpaired t-test: -RAP vs +RAP ($t=13.49$, d.f.=8, $p=0.0001$). Error bars show ± 1 standard deviation.

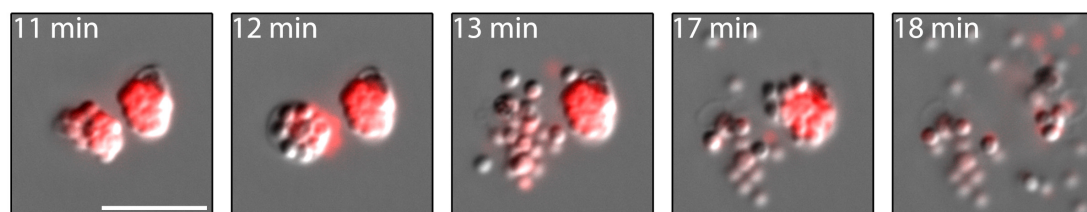


Figure 5.18: **Episomal expression of WT *SUB1* restores normal PVM rupture and merozoite release following disruption of the chromosomal *SUB1* locus.** Still images from time-lapse DIC and fluorescence microscopic analysis of a clone harbouring the WT *SUB1* complementation vector, isolated after RAP-induced disruption of the chromosomal gene in SUB1HA3:loxP clone 3E4 parasites. As can be seen, normal PVM rupture and merozoite release occurs. Presence of the episomal construct is indicated by expression of mCherry marker protein (red). Time from commencement of microscopic examination is shown. Scale bar, 10 μ m.

5.7 *SERA6* is not required for rupture of the PVM but is required for rupture of the erythrocyte membrane to achieve merozoite release.

To assess the importance of *SERA6* for schizont egress, live time-lapse microscopy was used to examine the egress of *SERA6:loxP* clones C4 and D2 at the end of cycle 1 following mock- or RAP-treatment of synchronous ring-stage parasites. This revealed very little merozoite release in the RAP-treated parasites, suggesting that *SERA6* is required for egress. In contrast to the Δ *SUB1* parasites, however, the Δ *SERA6* parasites were observed to successfully undergo rounding up and PVM rupture, as evidenced by the change in shape of the bounding erythrocyte membrane and increased visibility and motility of merozoites (Figure 5.19). This was interpreted as demonstrating that *SERA6* is required specifically for erythrocyte membrane rupture. To quantify this effect, erythrocyte membrane rupture and consequent merozoite release was scored and normalised in a similar fashion as for PVM rupture following *SUB1* disruption. This revealed that erythrocyte membrane rupture and merozoite egress was significantly higher in mock-treated parasites (mean = 89.8%, SD = 13.5, n = 10) compared to RAP-treated parasites (mean = 1.0%, SD = 2.3, n = 10). An unpaired two-sample t-test confirmed this difference to be highly significant ($t = 20.5$, d.f. = 18, $p < 0.0001$). Examination of a clone that was isolated following the RAP-treatment of *SERA6:loxP* clone C4 harbouring the WT *SERA6* complementation construct (confirmed to be excised at the chromosomal *SERA6* locus) revealed that normal egress had been restored to these parasites (Figure 5.20), confirming the role of *SERA6* in erythrocyte membrane rupture.

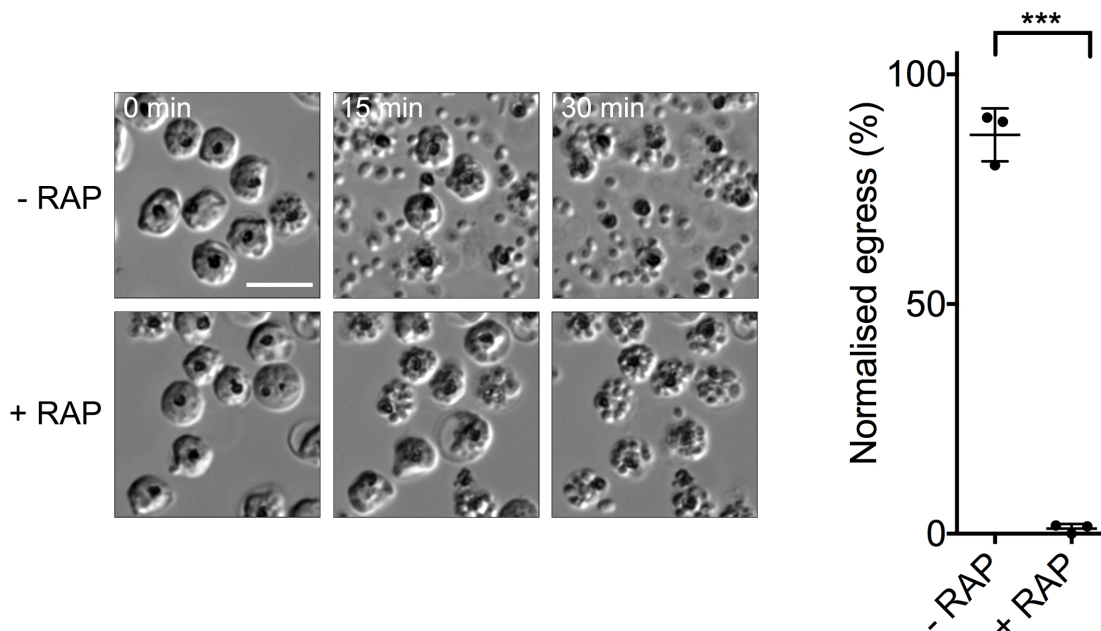


Figure 5.19: **Silencing of *SERA6* expression prevents erythrocyte membrane rupture.** Left-hand side, DIC images from time-lapse microscopy of mature *SERA6:loxP* clone schizonts. Parasites were mock- (- RAP) or RAP-treated (+ RAP) at ring-stage then Percoll purified at the end of cycle 1 and C2 arrested. C2 arrest was reversed by washing to allow egress to proceed and parasites immediately examined microscopically for 30 min. Time from commencement of microscopy is indicated. Scale bar, 10 μ m. In mock-treated samples merozoite release is clearly discernible, in contrast to RAP-treated parasites which undergo all of the usual morphological changes apart from erythrocyte membrane rupture and merozoite release. Right-hand side, quantification of erythrocyte membrane rupture and merozoite release revealed that significantly fewer RAP-treated *SERA6:loxP* parasites underwent merozoite release (mean = 1.0, SD 2.3, n = 10) compared to mock-treated parasites (mean = 89.8, SD = 13.5, n = 10). Data are collated from 10 videos each of mock- and RAP-treated parasites from 4 independent experiments (total number of observed egress events in mock-treated parasites, 356). Percentage egress of each replicate within a single experiment was normalised with the replicate that demonstrated the greatest percentage egress in a single experiment (100%). Statistical significance was determined by two-tailed unpaired t-test: -RAP vs +RAP (t = 20.46, d.f. = 18, p < 0.0001). Error bars show \pm 1 standard deviation.

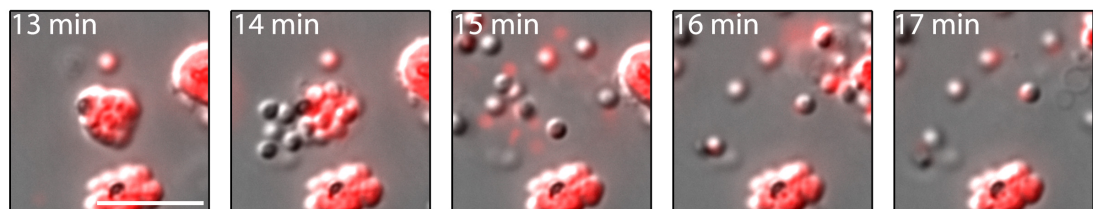


Figure 5.20: **Episomal expression of WT *SERA6* restores normal erythrocyte membrane rupture and merozoite release following disruption of the chromosomal *SERA6* locus.** Still images from time-lapse DIC and fluorescence microscopic analysis of a clone harbouring the WT *SERA6* complementation vector, isolated after RAP-induced disruption of the chromosomal gene in *SERA6:loxP* clone C4. As can be seen, normal merozoite release occurs. Presence of the episomal construct is indicated by expression of mCherry marker protein (red). Time from commencement of microscopic examination is shown. Scale bar, 10 μ m.

5.8 Fluorescent tagging of the PVM protein EXP1 confirms that PVM rupture and erythrocyte membrane poration occur in Δ *SERA6*, but not Δ *SUB1* parasites.

The DIC imaging presented above provided insights into the physico-mechanical processes that take place following disruption of *SUB1* or *SERA6*. A particularly interesting step in the egress pathway is that at which the intracellular merozoites suddenly become more easily discernible (associated with loss of different interference contrast of the images) and mobile. In agreement with previous work from this group [C. R. Collins, Hackett, et al. 2017], this was observed to occur concomitantly with erythrocyte rounding-up and was initially interpreted to represent the point at which the PVM ruptures, leaving free merozoites bound only by the erythrocyte membrane. To unambiguously ascertain whether PVM rupture occurs in Δ *SERA6* but not Δ *SUB1* schizonts, and whether this change in DIC imaging indeed corresponds to PVM rupture, SUB1HA3:loxP clone 3E4 and SERA6:loxP clone C4 were transfected with an episomal construct designed to express the PVM resident protein EXP1, fused at the C-terminus to the fluorescent reporter protein mCherry [Graewe et al. 2011]. Time-lapse microscopy of these lines in the presence of fluorescent wheat germ agglutinin which binds to glycoproteins on the exterior surface of the erythrocyte membrane allowed discrimination of the two membranes by fluorescence microscopy. As described in the Introduction, shortly before erythrocyte membrane rupture and merozoite release, the erythrocyte membrane becomes permeable. This can be visualised using the F-actin binding peptide phalloidin, which binds the short actin filaments that are integral to the erythrocyte cytoskeleton. It has been shown that fluorescently labelled phalloidin is only able to traverse the erythrocyte membrane when it becomes permeabilised. When it does so it binds to the F-actin in the cytoskeleton producing a local high concentration of phalloidin around the internal periphery of the erythrocyte which can be visualised microscopically [Glushakova, Humphrey, et al. 2010]. Time-lapse combined DIC and fluorescent microscopic imaging of the EXP1:mCherry tagged parasite lines in the presence of fluorescently labelled wheat germ agglutinin and fluorescently labelled phalloidin clearly confirmed that rounding up, PVM rupture and erythrocyte membrane poration occurred normally in Δ *SERA6* parasites as in mock-treated control parasites, but that none of these events occurred in Δ *SUB1* parasites (Figure 5.21). This experiment demonstrated that erythrocyte membrane poration occurs only after PVM rupture. Raising the possibility that the factor responsible for poration is released from the PV lumen or PVM when the PVM ruptures. It also confirmed that the point at which merozoites become readily discernible by DIC imaging is indeed the point at which PVM rupture has occurred.

5.9 Transmission and scanning electron microscopy confirm that rounding up of the parasitised erythrocyte and PVM rupture occurs following disruption of *SERA6*, but not following disruption of *SUB1*.

To corroborate the above findings, transmission and scanning electron microscopy (EM) was used to examine the internal and external morphology of mock- and RAP-treated *SUB1HA3:loxP* clone 3E4 and *SERA6:loxP* clone C4 schizonts. Transmission EM demonstrated that the PVM of Δ *SUB1* parasites was intact, as in C2 arrested parasites (Figure 5.22). Scanning EM demonstrated rounding up of Δ *SERA6* parasites following C2 release (Figure 5.22). Transmission EM demonstrated PVM rupture had occurred in Δ *SERA6* parasites, mimicking E64 arrest following C2 wash-off (Figure 5.22). The single bounding membrane that could be seen surrounding Δ *SERA6* and E64 arrested parasites contained knob structures demonstrating it to be the erythrocyte membrane. These findings corroborate what was seen by light microscopy, presented in Section 5.8.

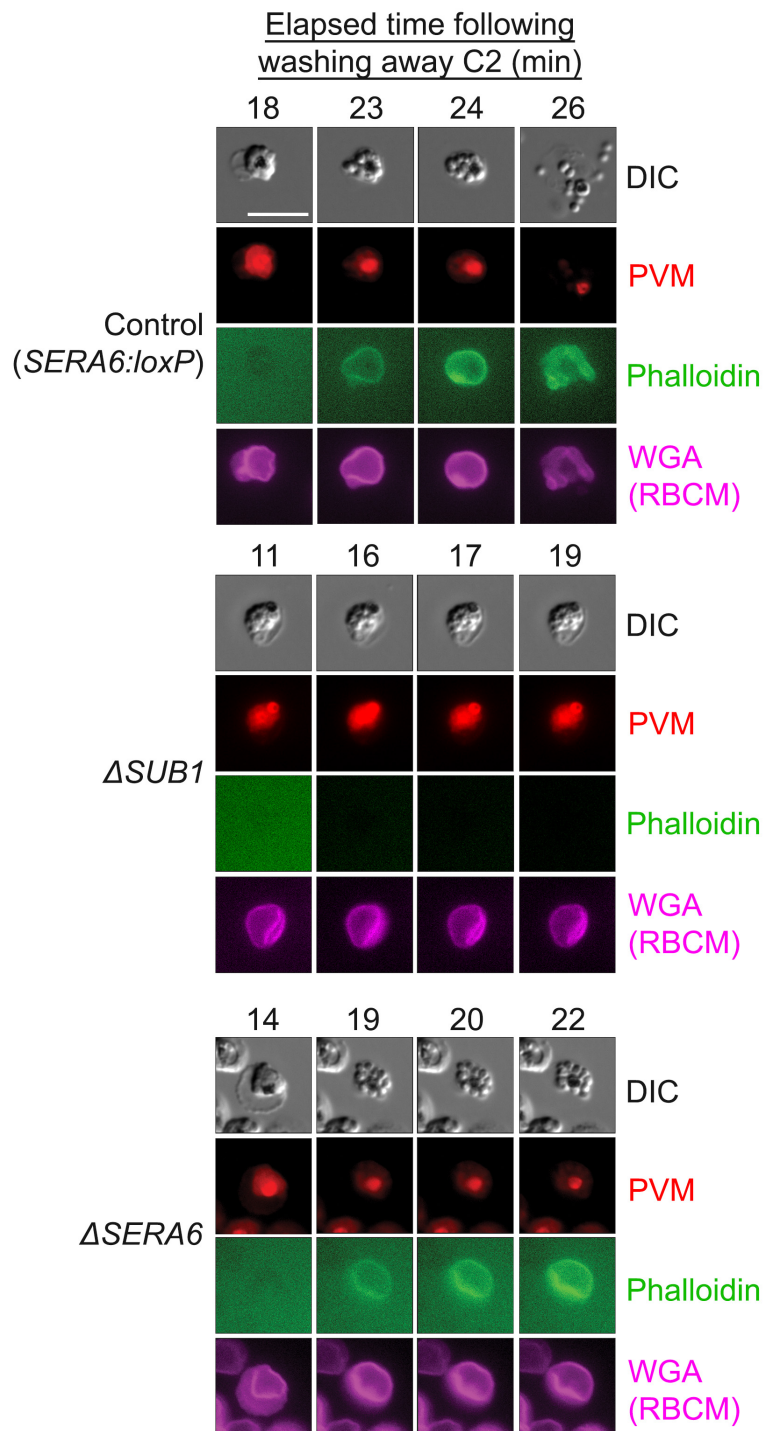


Figure 5.21: Rounding up, PVM rupture and erythrocyte membrane poration occur normally in Δ *SERA6* schizonts but not Δ *SUB1* schizonts. Images from simultaneous time-lapse DIC and fluorescence microscopic examination of control (mock-treated *SERA6:loxP*), Δ *SUB1* and Δ *SERA6* schizonts harbouring an episomal construct to express an EXP1:mCherry fusion to delineate the PVM. Schizonts were examined in the presence of fluorescently labelled phalloidin, which indicates the point at which erythrocyte membrane poration occurs by binding to F-actin in the host cell cytoskeleton, and wheat germ agglutinin (WGA) which binds to and delineates the outer surface of the erythrocyte membrane. Times following wash-off of the egress inhibitor C2 are shown. PVM swelling and rupture as well as erythrocyte membrane poration occurs in Δ *SERA6* parasites but not in Δ *SUB1* parasites, whilst erythrocyte membrane rupture and merozoite release occurs in neither mutant.

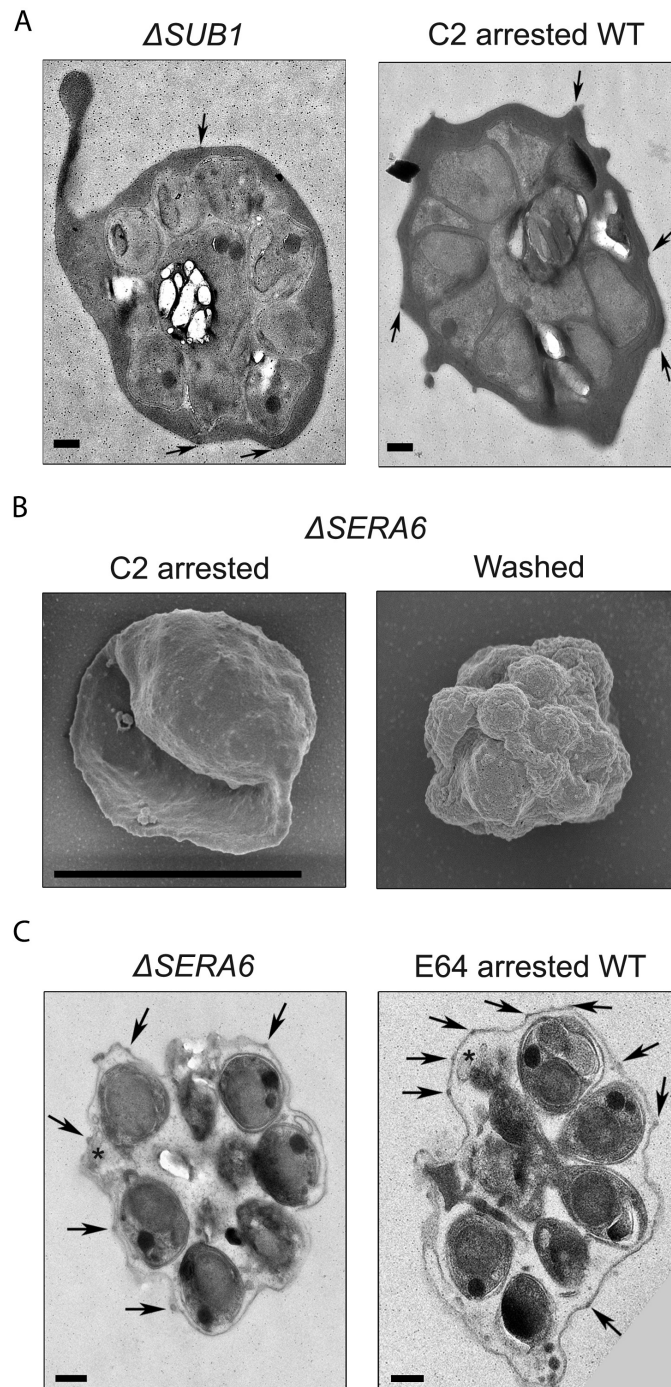


Figure 5.22: Electron microscopy confirms that PVM rupture and morphological changes associated with egress other than erythrocyte membrane rupture do occur in $\Delta SERA6$ parasites but not in $\Delta SUB1$ parasites A) Transmission EM confirms that $\Delta SUB1$ parasites ($\Delta SUB1$) are bound by an intact PVM. This is also the case with parasites in which egress is inhibited by C2 treatment (C2 arrested WT). Scale bar, 1 μ m. B) Scanning EM demonstrates that $\Delta SERA6$ parasites have undergone rounding up following wash-off of C2. Scale bar, 10 μ m. C) Transmission EM confirms that PVM rupture, as seen by the increased spacing of merozoites and the lack of an intact PVM occurs in $\Delta SERA6$ parasites ($\Delta SERA6$) as in E64 arrested parasites (E64 arrested WT). Scanning electron microscopy was carried out in collaboration with Gema Vizcay-Barrena in the laboratory of Roland Fleck, and transmission electron microscopy was carried out in collaboration with Victoria Hale, Claudine Bisson and Trishant Umrekar in the laboratory of Helen Saibil.

5.10 Surrogate markers of maturation reveal that $\Delta SUB1$ and $\Delta SERA6$ parasites develop normally following PKG activation.

5.10.1 AMA1 discharge and re-localisation from micronemes to the merozoite surface in $\Delta SUB1$ parasites confirms that merozoites progress normally following PKG activation despite not rupturing the PVM.

As presented, the phenotype of $\Delta SUB1$ schizonts was indistinguishable from that observed in C2 arrested schizonts, with no signs of rounding up or rupture of either bounding membrane. To assess what cellular processes might be occurring in the $\Delta SUB1$ parasites, localisation of the micronemal protein AMA1 was examined by IFA following C2 wash off. AMA1 is known to be rapidly discharged from micronemes onto the merozoite surface following PKG activation [C. R. Collins, S. Das, et al. 2013], an event that is easily visualised by IFA as the staining pattern transforms from a punctate (micronemal) signal to a circumferential, merozoite surface pattern similar to that of the GPI-anchored merozoite surface protein MSP1. Such a transformation was seen in $\Delta SUB1$ parasites following reversal of C2 arrest by wash-off, indicating that microneme discharge occurred normally (Figure 5.23). This demonstrates that mature $\Delta SUB1$ parasites are biochemically active and not merely developmentally arrested in some other fashion. It also demonstrates that microneme discharge alone is insufficient for PVM or erythrocyte membrane rupture to occur. No markers of exonemes other than SUB1 are known and so it was not possible to directly assess whether exonemes are discharged normally in $\Delta SUB1$ parasites. However, given that micronemes are clearly discharged normally following reversal of PKG inhibition, and that SUB1 is known to be discharged from exonemes concomitantly with AMA1 discharge from micronemes [C. R. Collins, S. Das, et al. 2013] it is reasonable to assume that exoneme discharge also occurs normally in $\Delta SUB1$ parasites.

5.10.2 AMA1 discharge and re-localisation from micronemes to the merozoite surface, as well as normal SUB1-mediated processing of MSP1 and SERA5 confirms that $\Delta SERA6$ parasites mature normally following activation of PKG.

In a similar fashion to that outlined above, the maturation of $\Delta SERA6$ parasites was assessed by a combination of IFA to examine whether microneme discharge occurred, and western blot analysis to examine whether SUB1-mediated proteolytic processing of MSP1 and SERA5 occurred. Microneme discharge was apparent from the re-localisation of AMA1 following C2 wash off (Figure 5.24). To examine SERA5 and MSP1 processing, mock- or RAP-treated schizonts of SERA6:loxP clone C4 were arrested with C2. The schizonts were then washed into Albumax II-free medium containing either fresh C2, E64 in the absence of C2, or no drugs and incubated for 30 min. Pelleted parasite material and culture supernatants were then harvested and fractionated by SDS-PAGE. Western blot analysis demonstrated clear SUB1-mediated processing of MSP1 and SERA5, demonstrating that the maturation of merozoites that normally occurs in preparation for their

release from the erythrocyte proceeds in the absence of SERA6.

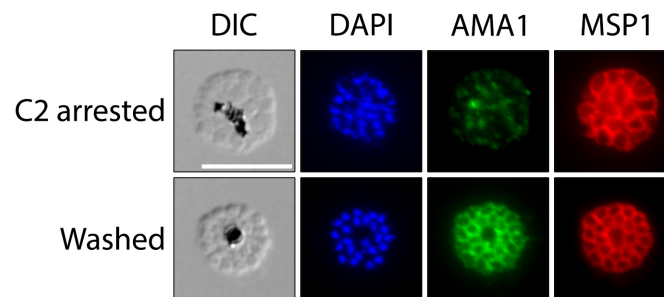


Figure 5.23: **Microneme discharge takes place in arrested Δ *SUB1* parasites.** IFA demonstrating that normal discharge of micronemes occurred in RAP-treated *SUB1HA3:loxP* clone 3E4 as apparent from visualisation of AMA1 re-localisation to the surface of trapped intracellular merozoites. Parasites were mock (- RAP) or RAP-treated (+ RAP) at ring-stage then Percoll purified at the end of cycle 0 and C2 arrested. C2 arrest was reversed to allow egress to proceed by wash-off and parasites incubated for 5 min before thin films were made on glass slides. Staining was performed with α -AMA1 polyclonal rabbit serum and the MSP1-specific mAb X509. DNA was stained with DAPI. Scale bar, 10 μ m.

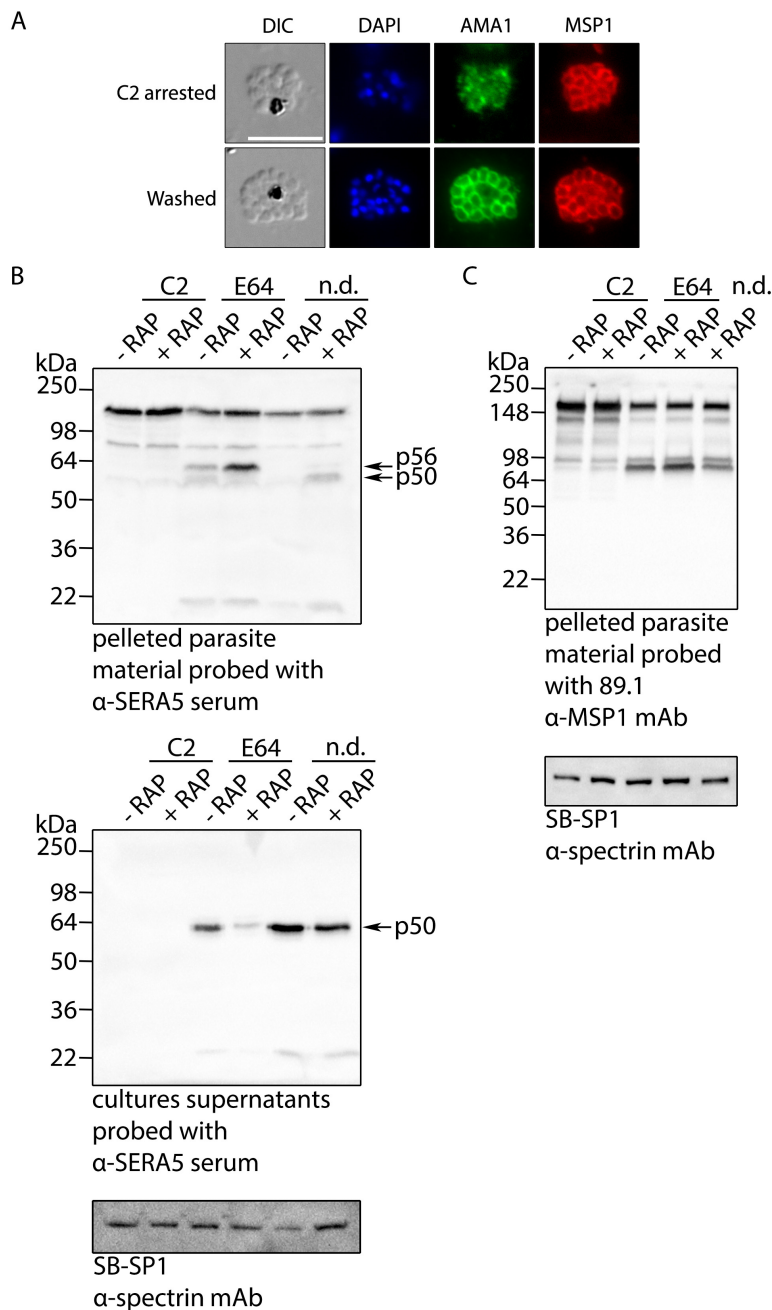


Figure 5.24: Normal microneme discharge and SUB1 activity in arrested Δ SERA6 parasites. A) IFA analysis demonstrating that normal discharge of micronemes occurs in RAP-treated SERA6:loxP clone C4 as apparent from visualisation of AMA1 re-localisation to the surface of trapped merozoites. Parasites were mock- (- RAP) or RAP-treated (+ RAP) at ring-stage then Percoll purified at the end of cycle 1 and C2 arrested. C2 arrest was reversed to allow egress to proceed by wash-off and parasites incubated for 5 min before thin films were made on glass slides. Staining was performed with α -AMA1 polyclonal rabbit serum and counterstaining with the α -MSP1 mAb X509. DNA was stained with DAPI. Scale bar, 10 μ m. B) Western blot analysis of extracts from parasites. Mature C2 arrested schizonts as described in A) were washed in and re-suspended into Albumax II-free medium containing fresh C2, E64 in the absence of C2, or no drugs (n.d.), and incubated for 30 min. Pelleted parasite material and culture supernatants were then harvested and examined by western blot, probed with an α -SERA5 polyclonal rabbit serum or the α -MSP1 mAb 89.1. Blots of the same samples were also probed with the α -spectrin mAb SB-SP1 as a control. Processed forms of SERA5 and MSP1 (indicated by arrows) were detected in mock- and RAP-treated samples.

5.11 SERA6 is not responsible for the additional, SUB1 independent processing that converts the p56 form of SERA5 to a p50 form.

As discussed in the Introduction, the SERA proteins expressed during the asexual blood stage cycle are processed by SUB1 either side of the papain-like domain. In the case of SERA5 this converts full length SERA5 (p120) to a p56 form. SERA5 has been found to undergo additional non-SUB1-mediated processing which then converts the p56 to a p50 form. The biological significance of this is unclear as is the identity of the protease that mediates this cleavage event. This unknown protease is referred to as 'protease X' and appears to be a cysteine protease due to its sensitivity to E64 [Debrabant et al. 1992; Stallmach et al. 2015]. This raised the intriguing possibility that SERA6 might be protease X and that at least one way in which SERA6 might act is via processing of SERA5. A subset of the samples presented in Figure 5.24 were fractionated on a single SDS-PAGE gel to compare the migration of the various SERA5 species. This confirmed that the p50 form of SERA5 was highly abundant in culture supernatants of both mock- and RAP-treated SERA6:loxP clone C4 schizonts that were incubated following reversal of C2 arrest by washing (Figure 5.25). The presence of the SERA5 p50 form in the culture supernatants of Δ SERA6 parasites clearly demonstrates that SERA6 is not responsible for the protease X activity that processes SERA5.

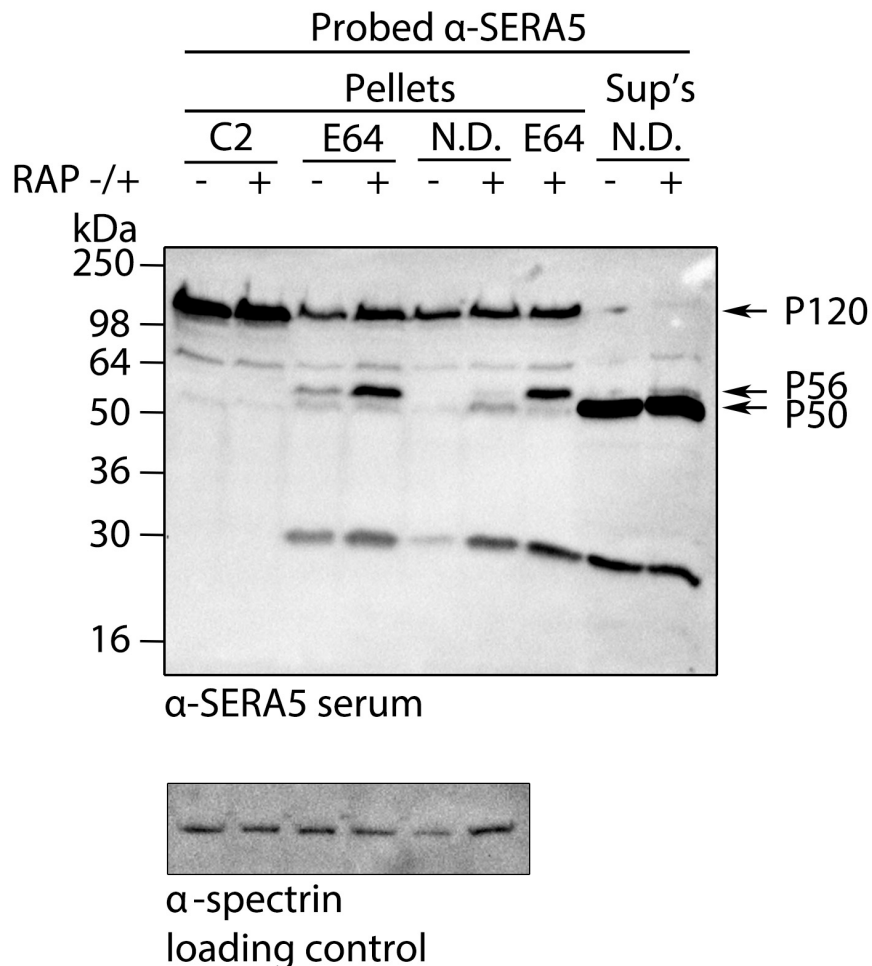


Figure 5.25: **SERA6 is not responsible for the protease X activity that processes SERA5 p56 to its p50 form.** Percoll purified schizonts of mock- (-RAP) and RAP-treated (+ RAP) cultures were arrested with C2 at the end of cycle 1. Schizonts were washed into Albumax II-free medium containing either fresh C2 (C2), E64 in the absence of C2, or no drugs (n.d.) followed by incubation for 30 min before pelleted parasite material and culture supernatants (sup's) were harvested and analysed by Western blot. Some samples have been loaded multiple times to aid comparison of fragment sizes. Different processed forms of SERA5 are indicated. A second blot of pelleted parasite material was probed with the α -spectrin mAb SB-SP1 as a loading control.

5.12 Shaking culture does not reverse the egress defects of $\Delta SUB1$ or $\Delta SERA6$ parasites.

Having observed the egress defects that manifest following disruption of *SUB1* or *SERA6*, it was natural to ask whether these defects might be overcome by mechanical liberation of merozoites. This was particularly the case for $\Delta SERA6$ parasites as anecdotal evidence suggests that these, like parasites arrested with E64 following C2 wash-off, are relatively fragile compared to C2 arrested or $\Delta SUB1$ parasites; when making thin smears for Giemsa staining it was noticed that both E64 arrested schizonts and $\Delta SERA6$ parasites would often rupture, resulting in a spread of merozoites not contained within a bounding membrane, whereas C2 arrested and $\Delta SUB1$ parasites reliably remained intact. In light of this, and the fact that disruption of either of these genes does not appear to inhibit normal maturation of merozoites it was reasoned that mechanical rupture of mature schizonts to liberate free merozoites might overcome the growth defects that act via blocks in egress. Mechanical liberation of free merozoites by syringe lysis [Dvorin et al. 2010] and by filtration [Boyle et al. 2010] has been described previously. Attempts were therefore made in preliminary experiments to liberate merozoites by passing mature schizonts mixed with fresh erythrocytes through a 28G needle. The invasion achieved through such approaches was found to be inefficient and inconsistent however, which prohibited this method from being used in a quantitative manner to compare invasion rates across different cultures. Instead a different approach was turned to. Normally *P. falciparum* is cultured under static conditions but gentle shaking is also routinely used during egress of mature schizonts in the presence of fresh erythrocytes, in order to obtain highly synchronous ring stage cultures with infected erythrocytes predominantly infected by single parasites. It was reasoned that shear forces generated by vigorous shaking might be sufficient to liberate invasive merozoites from arrested $\Delta SUB1$ or $\Delta SERA6$ schizonts. This was considered a particularly important issue because during natural infection parasitised erythrocytes are continuously exposed to the shear forces generated by passage through the circulation or (in the case of sequestered schizonts in the deep vasculature) impingement from passing uninfected erythrocytes. To test this notion, cultures containing mature schizonts ($\sim 1\%$ parasitaemia) of mock- or RAP-treated SUB1HA3:loxP clone 3E4 and SERA6:loxP clone C4 parasites were incubated either under standard stationary conditions, or with vigorous shaking (225 rpm) overnight, sampling both at the beginning of the experiment and the next day to count parasitaemias by flow-cytometry. The results demonstrated that for mock-treated parasites, shaking produced an approximately two-fold increase in parasite proliferation rate compared to stationary culture conditions, presumably due to more effective dispersal of merozoites (Figure 5.26). However, shaking conditions failed to significantly increase the poor proliferation rate of $\Delta SUB1$ or $\Delta SERA6$ parasites following overnight culture. While the exact magnitude of the shear forces created in this experiment are unknown (as are the shear forces exerted on sequestered schizonts by blood flow *in vivo*), this suggests that neither the $\Delta SUB1$ nor $\Delta SERA6$ defects would be readily overcome *in vivo*.

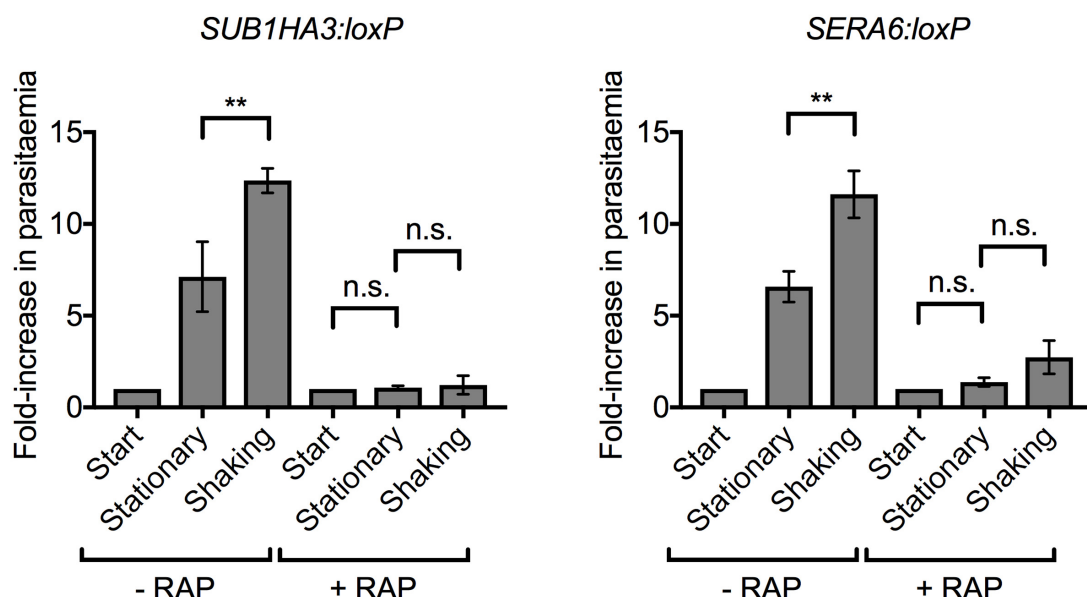


Figure 5.26: **Shaking fails to significantly increase the proliferation of Δ SUB1 or Δ SERA6 parasites.** Plots showing fold-change in parasitaemia overnight under stationary or vigorous shaking conditions. Starting cultures were established at $\sim 1\%$ (between 0.8% and 1.7%) following mock- (- RAP) or RAP-treatment (+ RAP). SUB1HA3:loxP cultures were established at the end of cycle 0, SERA6:loxP cultures at the end of cycle 1, following mock- or RAP-treatment. Data displayed here are normalised such that starting parasitaemia values are exactly 1% for each replicate experiment to allow comparison across experiments. Four replicates were performed for each gene disruption using both of the clones in each case. Two-sample t-tests were used to determine significance. T-test statistics for SUB1HA3:loxP: - RAP stationary vs - RAP shaking ($t = 5.233$, d.f. = 5, $p = 0.0034$) $n=4$. + RAP start vs + RAP stationary ($t = 1.722$, d.f. = 5, $p = 0.1456$) $n=4$. + RAP stationary vs + RAP shaking ($t = 0.4585$, d.f. = 5, $p = 0.6658$) $n=4$. T-test statistics for SERA6:loxP: - RAP stationary vs + RAP shaking ($t = 5.674$, d.f. = 4, $p = 0.0048$) $n=4$. + RAP start vs + RAP stationary ($t = 2.741$, d.f. = 4, $p = 0.0518$) $n=4$. + RAP stationary vs + RAP shaking ($t = 2.526$, d.f. = 4, $p = 0.0649$) $n=4$. Error bars show ± 1 standard deviation.

5.13 Conclusions

Both SUB1 and SERA6 have been hypothesised to be involved in *P. falciparum* blood stage egress for some time but the evidence for this has remained incomplete. Through the use of the conditional DiCre system this study has conclusively demonstrated their essentiality for the viability of asexual blood stage *P. falciparum* and elucidated the specific roles they play during egress. SUB1 is required for rupture of the PVM and all morphological changes that occur downstream of PKG activation. Intriguingly microneme discharge (and presumably exoneme discharge) occur normally. While SERA6 is not required for PVM rupture, rounding-up or erythrocyte membrane poration, it is required for the final event of erythrocyte membrane rupture. The failure to complement Δ *SEAR6* parasites with Cys⁶⁴⁴Ala mutant and uncleavable mutant forms of *SERA6* demonstrates that SERA6 functions as an enzyme and requires SUB1 processing. These data support a model whereby a bifurcating pathway leads to egress, with SUB1 acting as a central regulator of this process which mediates PVM rupture (directly or indirectly) and activates SERA6 which mediates erythrocyte membrane rupture (Figure 6.1). Erythrocyte membrane poration occurs immediately following PVM rupture and is independent of SERA6. It is unknown what mediates PVM rupture or erythrocyte membrane poration. The occurrence of erythrocyte poration in the presence of cysteine protease inhibitors [Glushakova, Humphrey, et al. 2010] demonstrates that calpain, or indeed other cysteine proteases, are not responsible for mediating it. Both PVM rupture and erythrocyte membrane poration are presumably mediated by either pore-forming proteins or phospholipases. It is possible that the poration factor is liberated from the PV into the residual erythrocyte cytoplasm. It is possible that it may be the same factor that mediates rupture of the PVM itself.

It has not been possible to determine whether exoneme biogenesis occurs normally in Δ *SUB1* parasites as the only known component of these organelles is SUB1 itself. As such it is conceivable that exonemes contain one or more additional components required for PVM rupture. It is possible that in Δ *SUB1* parasites exoneme biogenesis is adversely affected such that these other putative components are not discharged appropriately. However, this seems unlikely as microneme discharge, which occurs concomitantly with exoneme discharge was found to occur normally. It is unclear whether exonemes represent a truly distinct class of secretory organelles. Whilst they are clearly distinct from micronemes containing AMA1 [C. R. Collins, S. Das, et al. 2013] they may represent an additional sub-class of micronemes. In any case this study is the first to conclusively demonstrate the requirement of SUB1 for egress, including for activation of SERA6. Although prior evidence pointed to a role for SUB1 in egress [Yeoh et al. 2007] this relied on pharmacological inhibition using an inhibitory compound with potency in the micro molar range. As it was not possible in that study to determine the fine specificity of the inhibitor (for example by introduction of gatekeeper mutations that exclude the inhibitor from the enzyme in question), off-target effects may have been responsible for the phenotype observed. All the results obtained here, however, confirm and extend the findings of Yeoh and colleagues [Yeoh et al. 2007].

Chapter 6

Discussion

At the outset of this PhD project the physical and mechanical events of *P. falciparum* blood-stage egress were relatively well described. None of the molecular effectors responsible for mediating any of these events were known however. Over many years the accumulating genetic and biochemical evidence supported the hypothesis that both *SUB1* and *SERA6* are required for egress. Biochemical studies had shown that *SUB1* is a protease. *SUB1* was also shown to be secreted into the PV shortly before egress where it cleaves various parasite proteins including the putative papain-like protease *SERA6*. *SUB1* and *SERA6* were both found to be refractory to disruption and most significantly, pharmacological blockade of *SUB1* was found to block egress, although the inhibitor used (with an IC_{50} of 0.3 μ M) was not highly potent and significant off-target effects could not be excluded [Yeoh et al. 2007]. Nevertheless, the combination of evidence pointed to a role in egress for *SUB1* and *SERA6* and led to three hypotheses: 1) that *SUB1* is required for egress, 2) that *SERA6* is required for egress and 3) that *SERA6* functions as a protease, requiring *SUB1* processing for activation.

Egress is mediated by *SUB1* and *SERA6*.

It was with these hypotheses in mind that this PhD project was embarked upon. With the advent of the DiCre system for conditional gene modification in *P. falciparum* it was finally possible to address the above three hypotheses using a robust molecular genetic approach. Broadly speaking these hypotheses have been corroborated by the data obtained during this project. The data presented here demonstrate unambiguously that *SUB1* and *SERA6* are essential for *P. falciparum* egress during the asexual blood stage cycle *in vitro*. *SUB1* is required to mediate both breakdown of the PVM and all of the morphological changes that take place during egress following PKG activation (Figure 6.1). *SERA6* is not required for PVM rupture or any other morphological changes that occur during egress but is required for rupture of the erythrocyte membrane. The failure to complement Δ *SERA6* parasites with a catalytically dead mutant of *SERA6* or a form that cannot be cleaved by *SUB1* confirms that *SERA6* has an enzymatic role that depends on *SUB1* processing. Virtually all proteases are expressed as enzymatically inactive zymogen forms to prevent the potentially catastrophic consequences of uncontrolled proteolysis within a cell [Coulombe et al. 1996]. These findings are consistent with *SERA6* possessing protease activity but it cannot be said for certain, however, that it is a protease. The possibility that *SERA6* is a

non-peptidase protein with homology to the papain sub-family of cysteine proteases cannot be excluded. Examples of such non-peptidase proteins that resemble papain include pytochelatin synthase [Vivares, Arnoux, and Pignol 2005], arylamine N-acetyltransferase [Boukouvala and Fakis 2005], silacteins [Shimizu et al. 1998] and coagulation factor XIII transglutaminase [Makarova, Aravind, and Koonin 1999]. Definitive evidence that SERA6 is a protease, and that proteolytic activity is activated by SUB1 cleavage will likely require an *in vitro* biochemical demonstration of protease activity of purified SERA6 expressed recombinantly or pulled-down from the parasite. Such attempts are underway in the laboratory of Michael Blackman. A common mechanism by which the zymogen forms of proteases are kept inactive is by having an N-terminal prodomain, which is often required for correct folding of the translated protein, interact with the active site in order to prevent entry of any substrates, and thus their cleavage. This is the case for various protease of the papain clan (CA) including cathepsin B [Podobnik et al. 1997], cathepsin S [Vasiljeva et al. 2005] and papain itself [Roy et al. 2012]. Various proteases of clan CA cleave the prodomain from the mature portion of the enzyme auto-catalytically in an intramolecular fashion when activated by appropriate conditions (e.g. acidic pH) but others, such as cathepsin C and cathepsin X, rely on other proteases to remove their prodomains [Turk et al. 2012]. It is therefore likely that SERA6 is activated by SUB1 mediated cleavage of a prodomain portion of SERA6 from the active portion of SERA6, causing it to dissociate from the active site.

PVM rupture precedes rupture of the erythrocyte membrane.

The data presented here demonstrates that rupture of the PVM precedes rupture of the erythrocyte membrane and corroborates previous studies [Wickham, Culvenor, and Cowman 2003; Glushakova, Humphrey, et al. 2010; Glushakova, Busse, et al. 2017]. This has previously been a topic of some disagreement with an early study suggesting that erythrocyte membrane rupture occurs first [Salmon, Oksman, and Goldberg 2001]. The reason that Salmon and colleagues came to an opposing conclusion likely stems from the general toxicity of treating relatively early stage parasites with E64 as was performed in this study. This would likely be exacerbated if cultures were not highly synchronous. PVM rupture has also been shown to precede host cell membrane rupture of both liver-stage parasites [Burda, Caldelari, and Heussler 2017; Schmidt-Christensen et al. 2008; Putrianti et al. 2010; Graewe et al. 2011] and gametocytes [Sologub et al. 2011; Deligianni et al. 2013; Wirth, Glushakova, et al. 2014]. Thus, it appears the order of membrane rupture (PVM followed by erythrocyte membrane) is a consistent and universal feature of the *Plasmodium* lifecycle.

How is the sequential rupture of the two bounding membranes achieved?

The mechanism by which SUB1 mediates PVM rupture is uncertain. Unlike the erythrocyte cytoskeleton, the PVM is not known to possess structural protein components [Atkinson 1988]. It is therefore unlikely that this confirmed protease functions by cleaving structural protein components of the PVM or degrades lipid components of the PVM. It has not been possible to determine whether exoneme biogenesis occurs normally in $\Delta SUB1$

parasites as the only known component of these organelles is SUB1 itself. It is conceivable that exonemes contain one or more additional components that would be sufficient for PVM rupture but that these fail to be secreted into the PV. However, this seems unlikely as microneme discharge, which normally occurs concomitantly with exoneme discharge was found to occur normally. It is unclear whether exonemes represent a truly distinct class of secretory organelles. Whilst they are clearly distinct from micronemes containing AMA1 [C. R. Collins, S. Das, et al. 2013] they may represent an additional sub-class of micronemes. In any case this study is the first to conclusively demonstrate the requirement of SUB1 for egress, including for activation of SERA6.

Pore-forming proteins and phospholipases are used by various microbial pathogens for their egress from host cells [Friedrich et al. 2012b] and *P. berghei* liver stage schizonts have been shown to require a phospholipase, PbPL, for efficient PVM rupture [Burda, Roelli, et al. 2015]. Mature liver-stage $\Delta PbPL$ parasites either did not rupture the PVM or took significantly longer to do so. Intriguingly Burda and colleagues found that during liver stage infection PbPL localises to the PVM well before PVM rupture occurs. They suggested that PbPL is maintained at the PVM in an inactive state and requires activation through phosphorylation by kinases or processing by proteases. In line with this hypothesis it has been shown that a phospholipase C of *Listeria monocytogenes* is activated by proteolytic cleavage [Marquis, Goldfine, and Portnoy 1997]. At the time of publication Burda and colleagues [Burda, Roelli, et al. 2015] suggested that members of the SERA family might be responsible for activating PbPL, but given that work from the same laboratory (laboratory of Volker Heusseler) has since shown that PbSUB1 is required for rupture of the PVM during *P. berghei* liver stage infection [Burda, Caldelari, and Heussler 2017], and in light of the findings presented in this thesis, SUB1 would seem a more likely candidate. It was found that more than 50% of *PbPL* null parasites did eventually rupture the PVM however. This led the authors to suggest that redundancy of molecular effectors of PVM rupture exists, with multiple phospholipases and potentially other types of effectors, such as pore forming proteins, contributing to PVM rupture. Burda and colleagues found no defect during the erythrocytic stage of infection of $\Delta PbPL$ parasites but the possibility that another phospholipase, or that redundancy of function exists in this stage as well means the involvement of phospholipases in the rupture of the PVM during blood stage egress cannot be ruled out. Five putative phospholipases exist in the *P. falciparum* genome. One or more of these phospholipases, may be activated by SUB1-mediated proteolytic processing to mediate rupture of the PVM during asexual blood stage egress. Pore forming protein such as the PPLPs could also be involved with one or more of these contributing to PVM rupture. This might be achieved through osmotic pressure resulting from influx of water through a pore. TgPLP1, a perforin like protein, has been demonstrated to be involved in rupture of the *T. gondii* PVM [Kafsack et al. 2007] and this has been suggested to act by causing osmotic pressure which ruptures the PVM [Wade and Tweten 2015]. As outlined in the Introduction the current picture of PPLP involvement in egress is rather complex with studies carried out in different lifecycle stages and on orthologs in three different species. Further work will be required to determine the function of both the perforin and phospholipase genes in the stage-specific egress of individual species including *P. falciparum* blood stage egress. It is possible that these putative me-

diators of PVM rupture, hypothesised to be activated by SUB1 cleavage, are located in the micronemes/exonemes and discharged into the PV during egress, or they might already reside in the PV lumen at the point of SUB1 discharge.

Underlying the erythrocyte membrane is the mesh-like network of the cytoskeleton. This 'chicken-wire' like mesh is made up of α - and β -spectrin. Two heterodimers of entwined α - and β -spectrin associate to form a tetramer. Junctional complexes that consist of many components including band 3, Ankyrin and 4.1R tether the ends of spectrin tetramers into the membrane at either end with multiple spectrin tetramers attached to each junctional complex [Mohandas and Gallagher 2008]. Typically six spectrin tetramers are linked to each junctional complex which results in a hexagonal mesh. This cytoskeletal network is responsible for the remarkable properties of the human erythrocyte which include: tremendous elasticity (100x greater than that of latex), rapid responses to applied fluid stresses and a structural resistance greater than steel [Mohandas and Gallagher 2008]. This strength and resilience is required for the erythrocyte to withstand the considerable forces exerted upon it during its ~ 4 month existence in the bloodstream, with repeated passage through narrow capillaries with a cross section one third of the diameter of an erythrocyte and the fluctuations in pressure that this brings. In order for malaria parasites to achieve erythrocyte membrane rupture the cytoskeleton must therefore be disrupted in some fashion. This could involve the disruption of key protein-protein interactions of cytoskeletal components by the competitive interaction of a parasite protein, phosphorylation of cytoskeleton components to disrupt their interactions or proteolytic processing of cytoskeletal components to literally 'snip' components off one another. It has been proposed that a progressive dismantlement of the erythrocyte cytoskeleton occurs during parasite maturation [Millholland et al. 2011]. The findings presented herein and published recently [Hale et al. 2017] suggest that contrary to this claim, the erythrocyte cytoskeleton remains intact until very late in parasite development. Only following PKG activation which signals egress does rounding up and a collapse of the cytoskeleton appear to take place as seen by light microscopy and scanning electron microscopy.

Host calpain-1 in the erythrocyte has been implicated in mediating blood-stage egress through studies on resealed immuno-depleted erythrocytes [Chandramohanadas et al. 2009]. This demonstration that calpain-1, a cysteine protease, is involved in egress raises the question of whether it is responsible for mediating erythrocyte membrane rupture by degradation of the cytoskeleton and the physiologically relevant target of protease inhibitors, including E64 used in this and previous studies, that prevent egress. The genetic data presented here demonstrates that calpain-1 is not sufficient for egress and that egress does require the action of at least one confirmed parasite protease, SUB1, with SERA6 likely acting proteolytically as well. That the rodent malaria parasite *P. yoelii* was found to be viable in calpain^{-/-} mice also raises questions about the role of calpain-1 [Hanspal et al. 2002]. These conflicting data may occur due to differences between *in vitro* and *in vivo* egress, or biochemical differences between these two species. The data from this project demonstrate that in all ways examined E64 treatment following PKG activation mimics *SERA6* knockout, consistent with the idea that *SERA6* is the physiological relevant target of E64. Importantly though, this does not preclude the possibility that calpain-1 is indeed essential for egress and also a physiologically relevant target of E64

in the prevention of egress. It is possible that both SERA6 and calpain-1 act in concert to achieve erythrocyte membrane rupture and that E64 inhibition of either of these can prevent egress.

Supporting the idea that disruption of the erythrocyte cytoskeleton only occurs once egress is underway is as yet unpublished evidence from a colleague (Michele Ser Ying Tan in the laboratory of Michael Blackman). It has been demonstrated that cleavage of β -spectrin occurs following PKG activation, associated with egress. This discrete cleavage occurs between two calponin homology domains of β -spectrin. This is the region that associates with the junctional complexes and is apparently the only region of either α - or β -spectrin where cleavage would cause dissociation of the spectrin tetramers from the junctional complexes. This is because α - and β -spectrin associate with each other along the length of these molecules so discrete cleavage within these regions seems unlikely to affect their association. At this point it is unclear whether this processing is performed by SERA6 directly or by another protease but it is SERA6 dependent: cleavage of spectrin is not seen in cultures of Δ SERA6 parasites. A manuscript presenting a combination of data presented in this thesis with data outlining this spectrin cleavage has very recently received favourable reviews and is expected to be published in Nature Microbiology following the acquisition of some additional data. This raises two questions: what is the exact role of SERA6 in this process and is this cleavage sufficient for egress? With regards to the latter, there is not yet formal proof that this β -spectrin cleavage is sufficient for egress but it is highly likely that it is. Multiple hereditary human erythrocyte disorders result from mutations in the genes encoding cytoskeletal components including α - and β -spectrin. These disorders result in loss of erythrocyte membrane cohesion which leads to loss of membrane surface area and mechanical stability and causes changes to the shape of erythrocytes that defines various syndromes. They include: spherocytosis when erythrocytes take on a spherical shape; elliptocytosis when erythrocytes become ellipsoid in shape and pyropoikilocytosis where erythrocytes form in all shapes and sizes [Maillet et al. 1996]. In the case of spectrin, mutations have been identified that prevent spectrin dimer self-association [S. C. Liu, Palek, and Prchal 1982]. This results in large areas of the membrane being left free of spectrin coverage with the erythrocytes undergoing fragmentation. The symptoms associated with these syndromes vary from asymptomatic to severe and fatal haemolytic anaemia [Da Costa et al. 2013]. It is therefore entirely plausible that cleavage of spectrin that causes its dissociation from the membrane bilayer results in destabilisation of the erythrocyte membrane and its eventual rupture.

Given that cleavage of β -spectrin is SERA6 dependent one of two possibilities presumably occurs (assuming SERA6 is a protease): 1) SERA6 directly cleaves β -spectrin or 2) SERA6 activates another effector through proteolytic cleavage that achieves spectrin cleavage. The question of whether SERA6 directly cleaves spectrin will require a biochemical approach and will form the basis of further study in the laboratory of Michael Blackman. If the latter situation is true, it begs the following questions: what is cleaved by SERA6? Does the protein cleaved by SERA6 go on to cleave β -spectrin directly or does it act indirectly to achieve β -spectrin cleavage as well? The identification of SERA6 substrates will require proteomic analysis of Δ SERA6 and control parasites and will also be followed up in the laboratory of Michael Blackman. In any case this SERA6 dependent

cleavage of β -spectrin is highly likely to be the mechanism, or at least part of the mechanism, that achieves erythrocyte membrane rupture. Rupture of the erythrocyte membrane might also require the activity of other proteases upon components of the cytoskeleton. This might be the role that calpain-1 plays in achieving egress.

Regulated vesicle fusion, to achieve secretion of SUB1, is therefore central to *Plasmodium* egress and an understanding of how this is achieved following PKG activation is necessary for a complete understanding of egress. In *Toxoplasma gondii* TgDOC2.1 has been implicated in microneme secretion, resulting in both egress and invasion defects [Farrell et al. 2012]. TgDOC2.1 contains two C2 domains which are membrane binding domains found in a diverse range of eukaryotic proteins, most of which are involved in membrane trafficking and fusion by binding lipids in a calcium dependent manner [Corbalan-Garcia and Gómez-Fernández 2014]. Knockdown of the PfDOC2.1, the ortholog in *P. falciparum*, was found to result in an invasion defect concomitant with a decrease in the release of EBA-175 into the supernatant [Farrell et al. 2012]. No egress defect was observed suggesting that *Plasmodium* relies on microneme secretion for invasion but not for egress, and that exoneme secretion is sufficient for egress but requires different factors to those necessary for microneme secretion. As discussed in Section 1.13 calcium has been implicated in Apicomplexan egress including microneme secretion [Carruthers and L. D. Sibley 1999]. The identities of effectors that achieve exoneme secretion, and indeed the other effector molecules that are presumably involved in microneme secretion remain to be discovered.

The role of erythrocyte membrane poration during egress remains unknown.

It had been shown prior to this study, that shortly before merozoite release, the erythrocyte membrane becomes porated. It has also been demonstrated that the membrane ‘sealing’ molecules Tetronic 90R4 and P1107 interfere with exflagellation during gametocyte egress [Wirth, Glushakova, et al. 2014] and asexual blood stage egress [Glushakova, Humphrey, et al. 2010] respectively. In neither of these studies was the effect of these sealants on poration, or their general toxicity assessed however. The significance of this process remains unclear therefore and an assessment of its essentiality for, and role, in egress will require further work. Clearly poration alone is insufficient for egress to occur as porated parasites (arrested with E64) have been shown to remain intact by time-lapse microscopy [Glushakova, Humphrey, et al. 2010]. This has also been observed (not presented explicitly) during this study. The observation in this study that p50 SERA5 leaks from parasitised erythrocytes following rupture of the PVM demonstrates that large proteins, not just small molecules such as phalloidin, are able to traverse the erythrocyte membrane when poration occurs. It has also been observed (not presented here) that the p64 form of SUB1 leaks from E64 arrested parasites as well. Leakage of SERA6 has not been examined but it is reasonable to assume that the p65 form of SERA6 would also leak from the erythrocyte as well. It was also noticed during this study that culture supernatants of parasites treated with E64 following C2 wash-off were noticeably redder in colour compared to supernatants of parasite cultures where C2 treatment was maintained. This presumably results from the leaking of residual haemoglobin (64 kDa) from the parasitised cells. It is intriguing that at the point of erythrocyte membrane poration large proteins including key

molecular effectors of egress leak from the cell. They presumably cannot play any further role in the parasite lifecycle after this point. Of course, the arrest of porated parasites with E64 is an artificial situation and the leakage, indeed the poration itself, may merely be a side-effect of egress as the erythrocyte's rupture draws nearer. What causes erythrocyte membrane poration is unknown but could, as is hypothesised for PVM rupture, be a pore forming protein or phospholipase. Given that erythrocyte poration only occurs following PVM rupture it is tempting to speculate that it might be caused by exactly the same factor, or factors, that rupture the PVM and are then released into the confines of the erythrocyte. A molecule of haemoglobin is ~ 5 nm in width [Erickson 2009]. If a pore-forming protein is responsible for erythrocyte membrane poration it would have to create pores large enough to allow molecules of ~ 5 nm diameter to pass through them. Pores resulting from pore-forming proteins vary in diameter but have been described with diameters exceeding 5 nm so this is plausible [Parker2005]. Streptolysin-O forms pores that allow the passage of molecules with diameters of ~ 15 nm [Bhakdi and Sziegoleit 1985] and perfringolysin O can form pores of 150 nm diameter [Hong et al. 2002]. Interestingly, equinatoxin II which generally forms pores of 1-2 nm diameter has been shown to liberate haemoglobin from *P. falciparum* infected erythrocytes [Jackson et al. 2007]. This likely results from heterogeneity of pore size with some large enough to allow haemoglobin release. The authors report the existence of pores, as seen by scanning electron microscopy as large as 100 nm in diameter. Given what is understood of pore-forming proteins it is entirely plausible that the PPLPs (discussed in the Introduction and in this section) could be responsible for the poration of the erythrocyte membrane during egress.

What is the relevance of this work to real malaria infections?

During this project, I attempted to address the question of whether SUB1 and SERA6 would be required for egress during a natural infection. It might be hypothesised that *in vivo*, shear stresses from blood flow might mechanically rupture the erythrocyte membranes of $\Delta SUB1$ and $\Delta SERA6$ parasites, releasing viable merozoites that can successfully propagate. Previous studies have successfully achieved the release of viable invasion competent merozoites following mechanical rupture of the bounding erythrocyte membrane [Dvorin et al. 2010; Boyle et al. 2010]. In this study, however, these methods were not found to be efficient and consistent enough to allow a quantitative assessment of the viability of mature merozoites of mock- and RAP-treated cultures. It was therefore not possible to tease apart secondary effects of SUB1 or SERA6 knockout on merozoite invasion from the primary effects on egress, which almost certainly would affect invasion through preventing these labile forms from escaping the confines of an erythrocyte in a timely manner. The exposure of knockout parasites to the shear forces in vigorously shaking culture did not significantly reverse the growth defects that result from these gene knockouts. Of course, the shear forces that are exerted upon parasitised erythrocytes during shaking culture are unknown, as are the shear forces exerted upon schizonts in blood vessels. While the exact nature of a real infection cannot be mimicked it does suggest these defects are not readily overcome. To more definitively address this question studies would either have to be performed in monkeys using the DiCre system in *P. knowlesi*, which is currently being implemented (personal communication Robert Moon), or perhaps

using a flow culture system which more closely approximates the flow conditions of the bloodstream such as those used in studies of endothelial adhesion of infected erythrocytes [Ho et al. 1998; Cooke, Coppel, and Nash 2002]. Supporting the hypothesis that both *SUB1* and *SERA6* are essential to the parasite's viability during *in vivo* infection is the fact that the homologs of both of these genes appear refractory to knockout in *P. berghei*. Data from the PlasmoGEM project clearly demonstrates *SUB1* essentiality [Gomes et al. 2015]. *SERA6* knockout has yet to be performed in the PlasmoGEM system but knockout has been attempted on an individual basis and it was found to be refractory (personal communication Kai Matuschewski).

Further to this point, it is worth considering more generally what relevance the study of the eruptive, spontaneous *in vitro* egress of *P. falciparum* has to a real infection with non-laboratory strains of parasites. The fact that *P. berghei* does not undergo egress *in vitro* is often put forward as evidence that the eruptive egress seen in laboratory strains of *P. falciparum* might be an artefact of long-term *in vitro* culture. This feature of egress might have been selected for to achieve efficient dispersal of merozoites in static cultures and be unnecessary when parasites are present in the bloodstream which could rupture the bounding erythrocyte membranes and disperse merozoites by shear forces from blood flow. Supporting the idea that spontaneous eruptive egress is not an artefact of *in vitro* culture, however, is the observation that various different species and strains of *Plasmodium* appear to egress in this way including when freshly removed from an animal host. The first published video-microscopic examination of *Plasmodium* egress was of *P. knowlesi* which were freshly removed from a monkey and these were seen to egress spontaneously [Dvorak et al. 1975]. Similarly two strains of *P. yoelii* maintained in mice have been shown to egress in a similar fashion [Yahata et al. 2012]. Therefore spontaneous egress cannot merely be an artefact of *in vitro* culture, and actually seems to be a general feature of *Plasmodium* egress. It remains uncertain however whether egress of $\Delta SUB1$ or $\Delta SERA6$ parasites might nevertheless be achieved by mechanical rupture *in vivo*. It is unclear why the rodent malaria model *P. berghei* does not egress spontaneously *in vitro*. It is possible that it requires a host factor not present in *in vitro* culture or it might rely, to some extent, on shear forces of blood flow.

The use of the DiCre system.

The DiCre system has continued to be developed since its establishment and significant advances have been made since this PhD project was started. This means that were this project to be started again, some things would almost certainly be done differently. Indeed, the wide implementation of the DiCre system has both facilitated and necessitated innovation in complementary techniques, approaches and tools. The implementation of the *loxPint* by Jones and colleagues, and used in this project, was borne out of a desire to utilise the DiCre system more fully and effectively [Jones et al. 2016; Sherling et al. 2017]. The *loxPint* has and will be particularly important in realising the full potential of the DiCre system. It has allowed the floxing of gene fragments through the integration of single constructs, made integration of *loxP* sites more efficient by allowing targeting of integration into relatively GC rich coding sequences and allows enzymatic inactivation of genes by truncation, as was achieved for *SUB1* in this project. Indeed the analysis of *SERA6:loxP*

clones C4 and D2 in this study was made more difficult by the fact that the phenotype resulting from *SERA6* disruption only manifests after the completion of erythrocytic growth cycle 0. The maintenance of synchronous cultures of mock-treated and RAP-treated parasites for four days before analysis (~96 h post mock/RAP-treatment) is, if nothing else, laborious. It would be preferable to integrate a *loxPint* within the *SERA6* catalytic domain such that truncation of this putative protease domain would occur upon RAP-treatment. This would ablate the function of any residually expressed form of the gene on the resulting excised episome. The fact that *SERA6* was not truncated in this fashion and is present on an episome under control of its promoter is presumably responsible for the delayed Δ *SERA6* phenotype. The *loxPint* is also allowing the implementation of allele switching studies which are underway in the laboratories of Edgar Deu and Michael Blackman. The use of the *loxPint* for an allele switching strategy is also a preferable approach for genetic complementation compared to the episomal approach taken in this study. RAP-mediated excision could be used to swap a gene to an allele possessing specific mutations, or to reconstitute the wild-type gene. Chromosomal expression would likely achieve more stable and consistent expression of complementing alleles when 'switched on' compared to expression from episomal constructs which may vary due to episomal copy number. Although the *SUB1* and *SERA6* native promoter regions were used for episomal expression in this study inefficient expression, or an inappropriate profile of expression may have been responsible for the incomplete complementation seen. Allele switching would likely achieve expression profiles more closely resembling that of the endogenous wild-type gene in question as being in its normal genomic environment should place a complementing gene under control of endogenous cis-acting factors that regulate expression.

The way in which the DiCre system is being implemented has also changed since this project was embarked upon. The 1G5DC clone used in this study suffers from a significant drawback in that the DiCre expressing locus can revert to a wild-type locus meaning the parasite no longer expresses the DiCre fragments. This results from the fact that the DiCre expression cassette was integrated by single-crossover homologous recombination, creating a duplication of the targeting sequence. As this locus lacks a drug marker, following its excision from the genome [C. R. Collins, S. Das, et al. 2013], recombination can occur between the two identical targeting sequences. Reversion of this locus to wild-type likely cannot be undone and may be selected for if expression of the DiCre fragments are slightly costly to the parasite's fitness. Parasites not expressing the DiCre fragments will of course fail to excise DNA between *loxP* sites. A high proportion of parasites not undergoing excision when RAP-treated can confound the interpretation of data. This mandated the regular thawing of frozen parasite isolates. Even then, as reversion could 'spread' through the population in the culture either by selection or genetic drift after it had arisen, it was unpredictable. This required the periodic assessment of the prevalence of reverted parasites using PCR or an assay in which the phenotype was well established already. This has been a significant hindrance in working with clones derived from the 1G5DC clone. As such the DiCre fragment expressing locus of the 1G5DC clone has been modified using Cas9 genome editing technology to remove the displaced targeting sequence and prevent reversion (personal communication Christine Collins, laboratory of Michael Blackman). The DiCre fragment expressing locus in this new clone (B11DC) does appear

stable over long periods of time. Another DiCre expressing clone has also been created by integration of the DiCre expression cassette into the *pfs47* locus which is not essential during the asexual blood stage cycle [Knuepfer et al. 2017].

The plaque assay is a valuable addition to our suite of tools and technologies for malaria research.

It is from the use of the DiCre system and a desire to assay parasite replication at the level of individual parasites that created the impetus to develop the plaque assay presented herein. This was because, despite being highly efficient, the DiCre system results in heterogeneous populations of parasites following RAP-treatment. A small proportion of non-excised, effectively wild-type parasites, always remain and these replicate at exponential rates. This can confound the interpretation of results if analysis of RAP-treated populations is extended beyond a few growth cycles. Hopefully the simplicity, lack of complex and costly equipment and lack of expertise required to implement the plaque assay, as well as its scalability will facilitate studies of *Plasmodium* species, including in resource poor settings. The plaque assay is now routinely used in the laboratories of Michael Blackman, Edgar Deu and Moritz Treeck as well as others around the world for the isolation of parasite clones and assays of parasite growth in response to gene knockouts or inhibitor treatments. Colleagues in the laboratories of Edgar Deu and Moritz Treeck have developed semi-automated systems of plaque counting using Fiji software. The plaque assay has the potential to be scaled-up for medium or even high-throughput screens of parasite growth and automation of plaque counting would be an important step in implementation such projects. Furthermore, Robert Moon has and continues to develop a modified protocol to allow the plaque assay to be used for the assessment of *P. knowlesi* growth. It is therefore likely that the plaque assay will become a routinely used tool in malaria research.

In the last five years we have seen the implementation of some powerful and reliable tools for the manipulation of the malaria parasite genome including the DiCre system [C. R. Collins, S. Das, et al. 2013], Cas9 genome editing technology [Ghorbal et al. 2014], selection linked integration [Birnbaum et al. 2017] and artificial introns [Jones et al. 2016]. Combined with the advent of the *P. knowlesi* A1.H1 clone with its various advantages including high efficiency of genetic modification, larger merozoite dimensions and increased duration of merozoite viability [Moon, J. Hall, et al. 2013; Moon, Sharaf, et al. 2016; Tarr et al. 2014; Grüning et al. 2014] now is a particularly exciting time to be involved in research of the molecular and cellular biochemistry of the malaria parasite.

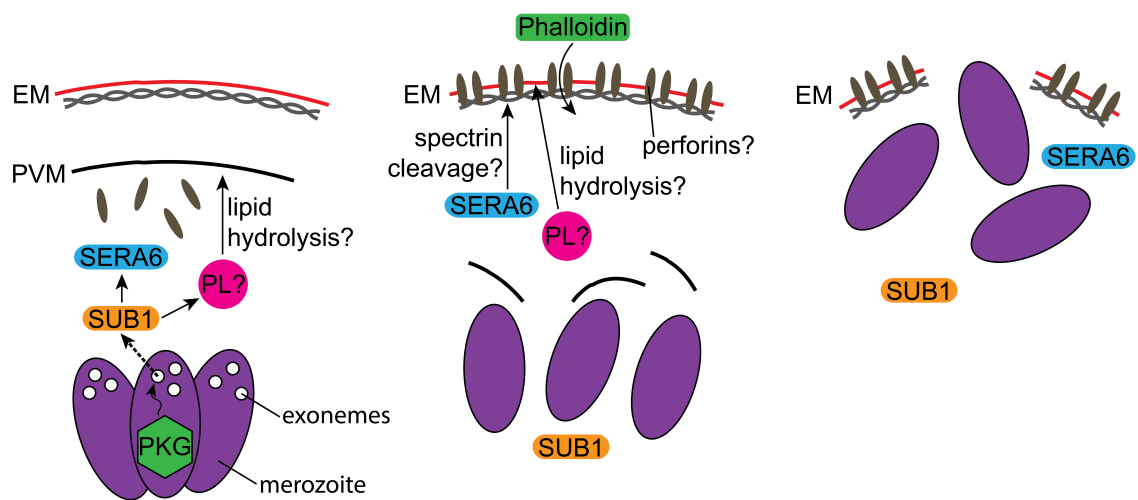


Figure 6.1: **Schematic model of the branching pathway leading to egress involving SUB1 and SERA6.** SUB1 is discharged from micronemes into the PV lumen following PKG activation and is required for PVM rupture, possibly by activating other effectors which might be perforins or phospholipases (PL?). SUB1 processes SERA6 either side of the papain-like domain and activates an enzymatic function. SERA6 is required for erythrocyte membrane rupture, perhaps by cleaving components of the cytoskeleton including β -spectrin, or activating other effectors through proteolytic processing which go on to cleave cytoskeletal components. The poration of the erythrocyte that occurs to allow phalloidin to enter the cell likely results from insertion of perforin molecules into the erythrocyte membrane. These putative perforins might be present in the PV and released into the residual erythrocyte cytosol upon PVM rupture, or might be released from exonemes or micronemes.

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Appendix: Publications arising from work presented in this thesis

RESEARCH ARTICLE

Development and Application of a Simple Plaque Assay for the Human Malaria Parasite *Plasmodium falciparum*

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OPEN ACCESS

Citation: Thomas JA, Collins CR, Das S, Hackett F, Graindorge A, Bell D, et al. (2016) Development and Application of a Simple Plaque Assay for the Human Malaria Parasite *Plasmodium falciparum*. PLoS ONE 11(6): e0157873. doi:10.1371/journal.pone.0157873

Editor: Tobias Spielmann, Bernhard Nocht Institute for Tropical Medicine, GERMANY

Received: March 31, 2016

Accepted: June 6, 2016

Published: June 22, 2016

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the United Kingdom Medical Research Council (<http://www.mrc.ac.uk/>) (U117532063 to MJB), the Francis Crick Institute (<https://www.crick.ac.uk/>), the Wellcome Trust (099950/Z/12/Z to ED and ISSF2 funding to the London School of Hygiene and Tropical Medicine) and European Commission Framework Programme 7 contract no. 242095 (EviMaLAR; <http://www.evimalar.org/>). JAT is in receipt of a Francis Crick Institute PhD studentship whilst SD was funded by an EviMaLAR PhD studentship. The funders had no role in study

Abstract

Malaria is caused by an obligate intracellular protozoan parasite that replicates within and destroys erythrocytes. Asexual blood stages of the causative agent of the most virulent form of human malaria, *Plasmodium falciparum*, can be cultivated indefinitely *in vitro* in human erythrocytes, facilitating experimental analysis of parasite cell biology, biochemistry and genetics. However, efforts to improve understanding of the basic biology of this important pathogen and to develop urgently required new antimalarial drugs and vaccines, suffer from a paucity of basic research tools. This includes a simple means of quantifying the effects of drugs, antibodies and gene modifications on parasite fitness and replication rates. Here we describe the development and validation of an extremely simple, robust plaque assay that can be used to visualise parasite replication and resulting host erythrocyte destruction at the level of clonal parasite populations. We demonstrate applications of the plaque assay by using it for the phenotypic characterisation of two *P. falciparum* conditional mutants displaying reduced fitness *in vitro*.

Introduction

As one of the most important human pathogens, much research on the human malaria parasite *Plasmodium falciparum* focuses on the identification and characterisation of drug targets and/or an improved understanding of how host immune responses interfere with parasite replication and associated pathology. During the clinically relevant asexual blood stage of the parasite lifecycle, merozoites invade host erythrocytes where they divide within a parasitophorous vacuole to produce 16–20 daughter merozoites. These are then released from the erythrocyte, completely destroying it in the process. In certain regards the parasite blood-stage lifecycle therefore mimics a viral lytic cycle, in that destruction of each host cell allows the release of multiple invasive forms which go on to invade and destroy further host cells, amplifying the

design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

pathogen population. For many viruses, this lytic cell cycle has long been exploited in *in vitro* assays in which the concentration of infectious viral particles in a sample can be determined by microscopic visualisation of destruction of host cells following their infection by suitably titrated aliquots of virus. First described for animal viruses by Dulbecco and Vogt in 1953 [1], the assay protocol usually involves limiting diffusive dispersion of the released viral particles through the use of semi-solid media in order to achieve discrete, highly localised regions of host cell monolayer destruction called plaques. The cell monolayers are finally stained to visualise the plaques. Because of their simplicity and broad applicability, plaque assays are amongst the most valuable and widely-used tools in viral research, allowing facile quantitation of the effects on viral replication of environmental conditions, drugs, antibodies and genetic manipulation, and simplifying isolation of viral clones. Plaque assays have also been developed for other intracellular pathogens, including several bacterial species [2] and even protozoan organisms related to the malaria parasite, notably *Toxoplasma gondii* which readily infects most nucleated mammalian cells and so can be cultured in adherent fibroblast monolayers [3]. In contrast, blood stages of *P. falciparum* and other *Plasmodium* species pathogenic to humans replicate exclusively in erythrocytes (or reticulocytes), which are not normally adherent. Plaque assays developed for *Plasmodium* have therefore used monolayers of erythrocytes adhered to the base of plastic tissue culture wells using concanavalin A [4, 5], Cell-Tak [6], or anti-Rhesus D antibodies plus protein L [7], with plaque formation being visualised using either Giemsa staining of fixed monolayers or immunofluorescence. Such assays were key to the success of elegant pioneering experiments demonstrating the phenomenon in which all the merozoite offspring of a single infected erythrocyte are committed to either continuation of the asexual life cycle or transformation into either male or female forms of the sexual stages (gametocytes) responsible for transmission to the mosquito vector [4, 5, 7]. However, due to the single-cell-thick nature of the adherent erythrocyte monolayers produced by these methods and the need for fixation and staining to visualise the plaques, the assays are unsuitable for routine quantitation of malaria parasite growth rates. Here we describe the optimisation and application of an extremely simple plaque assay that we expect will become an attractive and widely used addition to the available repertoire of malaria research tools.

Results

P. falciparum Growth in Static Erythrocyte Cultures Produces Plaques

In initial work, asexual blood-stage cultures of *P. falciparum* (clone 3D7) were dispensed in complete medium into the central 60 wells of flat-bottomed 96-well microplates and incubated undisturbed (without replacing the medium or disturbing the erythrocyte layers) at 37°C in sealed, humidified gassed chambers, monitoring by daily examination with an inverted light microscope. This revealed the gradual appearance and expansion of translucent, roughly circular discontinuities or apparent zones of clearance in the otherwise homogeneous erythrocyte layer coating the base of each well (Fig 1A). These discontinuities are henceforth referred to as plaques. Importantly, plaque formation was easily detected and recorded without opening the plates using a high resolution flatbed digital scanner (top-down transmission light mode, 4,800 dpi), avoiding the need for frequent and laborious microscopic examination of individual wells and also allowing simple recording and documentation of the plate images. To formally establish whether plaque formation was a result of parasite replication, synchronous parasite cultures (~10% parasitaemia, 0.75% haematocrit) were serially diluted into a suspension of fresh uninfected erythrocytes (0.75% haematocrit) in complete medium and aliquots of each dilution dispensed across a microplate. Monitoring of plaque formation in these plates showed that the mean frequency of plaques at any given dilution correlated linearly with the starting

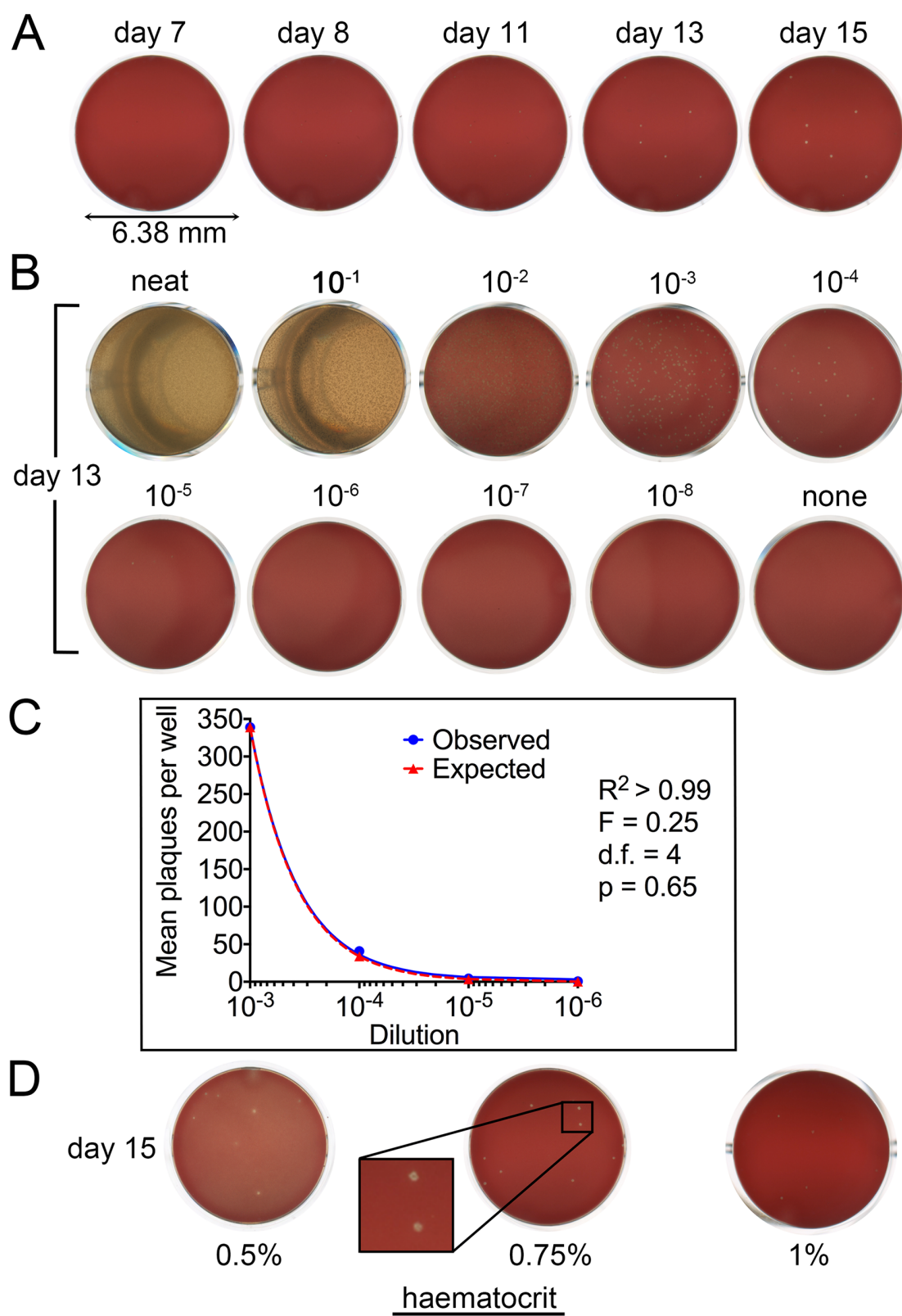


Fig 1. Formation of plaques in static *P. falciparum* asexual blood stage microplate cultures. (A) Time-dependent evolution of plaques. Scanned images (RGB) of a single microplate well (diameter 6.38 mm) taken at the indicated times following the introduction of a low parasitaemia *P. falciparum* 3D7 culture (200 μ l per well, 0.75% haematocrit, starting parasitaemia ~0.00004%, corresponding to ~6 infected cells per well). Plaques first became detectable by light microscopy or high resolution scanning at day 8. (B) Plaque density correlates with starting parasitaemia. Shown are microplate wells containing 10-fold serial dilutions (indicated) of a *P. falciparum* culture (0.75% haematocrit, starting parasitaemia 13%). The wells were imaged on day 13 following initiation of the culture. By this point, at the highest parasite densities (neat and 10^{-1} dilution) the erythrocyte layer was completely destroyed, whilst discrete plaques were visible at the lower parasite densities. Total numbers of plaques in each set of 6 replicate wells of the 10^{-3} – 10^{-8} dilutions (replicate wells are not shown) was 2,034 (10^{-3}), 245 (10^{-4}), 26 (10^{-5}), 4 (10^{-6}), and 0 plaques (10^{-7} and 10^{-8}). No plaques were ever detected in the complete absence of parasites (none). (C) Linear regression by analysis of covariance (ANCOVA) indicating a strong linear inverse correlation between dilution and plaque density in the wells containing the 10^{-3} – 10^{-6} parasite dilutions. A plot of observed mean plaque frequency against dilution (blue) from the experiment shown in (B) is shown alongside a plot of the plaque frequencies expected if there is a linear inverse correlation between plaque number and dilution (red). Values of the statistical data (R^2 , F statistic, number of degrees of freedom and p value) are shown. (D) Optimisation of haematocrit conditions. Microplate wells containing cultures at ~0.00004% parasitaemia at the indicated haematocrits, imaged on day 15. Whilst plaque formation was easily detected in the 0.75% haematocrit wells, they were much more difficult to detect at higher haematocrit, whilst at lower haematocrit values the plaques were typically more diffuse with signs of erythrocyte lysis. Inset, zoomed region of the 0.75% haematocrit erythrocyte layer, showing the discrete nature of the plaques.

doi:10.1371/journal.pone.0157873.g001

parasitaemia in each well (Fig 1B and 1C and S1 Fig). No plaques formed in the absence of parasites. These results suggested that, so long as the erythrocyte cultures remained static and undisturbed, parasite growth with concomitant host cell lysis was capable of producing restricted, microscopically discernible zones of erythrocyte destruction. The discrete nature of the plaques was presumably a result of repeated cycles of preferential invasion and lysis of immediately adjacent host cells upon completion of each erythrocytic growth cycle. To optimise conditions for plaque formation and detection, further assays were performed using a range of haematocrit values. This revealed that a haematocrit of 0.75% ($\sim 7.5 \times 10^7$ erythrocytes per ml, or $\sim 1.5 \times 10^7$ cells per well) resulted in optimal plaque morphology and clarity (Fig 1D). Interestingly, erythrocyte longevity under the conditions of the assay was also haematocrit-dependent, with clear signs of generalised erythrocyte lysis (i.e. appearance of free haemoglobin in the medium) typically evident as early as 10 days at a 0.5% haematocrit, whilst at higher haematocrits the cells were reproducibly completely stable for at least 2 weeks. All subsequent work therefore used a haematocrit of 0.75%. Typically, plaques attained a maximum diameter of ~0.1 mm before eventual complete lysis of all the erythrocytes between 16–21 days. Note that this was not due to excessive parasitaemia in the microwell cultures; microscopic examination of Giemsa-stained cultures recovered from wells containing 30 plaques at 14 days revealed parasitaemia values of only ~0.06% (~1 parasite in 1600 erythrocytes examined).

Each *P. falciparum*-Infected Erythrocyte in the Starting Population Forms One Plaque

We reasoned that during parasite growth in the environment of a microplate well, even under the static conditions described above, diffusive movement of free merozoites or even infected erythrocytes might allow multiple plaques to form from a single parental infected cell. To investigate this possibility, a synchronous parasite culture at ring-stage was diluted into a suspension of fresh erythrocytes at 0.75% haematocrit in order to obtain a theoretical density of <5 parasitised erythrocytes per ml of culture. The suspension was dispensed into the central wells of 4 flat-bottomed plates (200 μ l/well, 240 wells total), then the resulting plaques enumerated 15 days later. If the parasitised cells assort randomly and if each parasite-infected cell produces only a single discrete plaque, the numerical distribution of plaques across the wells is expected to follow Poisson statistics. A key indicator of this is that the experimentally determined (observed) mean average number of plaques per well is sufficient to allow a prediction

Table 1. Poisson analysis of plaque formation shows that each plaque derives from a single parasite-infected erythrocyte.

Plaques/well	Plaque frequency	
	Observed ^a	Expected ^c
0	168	163
1	56 ^b	63
2	11 ^b	12
3	5 ^b	2

^aPlaque number in a total of 240 wells (4 microwell plates).

^bThe total number of plaques in the four plates was 93, so the overall mean number of plaques/well (λ) is given by $93/240 = 0.388$.

^cExpected values assuming a Poisson frequency distribution. A chi-squared goodness of fit comparison of the observed and expected data gave a value $\chi^2 = 1.25$ for 2 degrees of freedom, $p > 0.50$, indicating no significant difference between the sets of values. Note that for the chi-squared calculations, data for the 2 plaques/well and 3 plaques/well were pooled in order to avoid using expected values of < 5 .

doi:10.1371/journal.pone.0157873.t001

of the probability distribution of plaque numbers across all the wells. In the experiment depicted in Table 1, the observed frequency distribution of plaques per well was compared to the theoretical Poisson distribution with the same mean number of plaques per well as observed in this experiment (0.388). A chi-squared test of goodness-of-fit showed that the observed distribution of plaques per well was not significantly different from the expected Poisson distribution. Similar data were obtained from three additional independent plaque assays with widely varying mean values of plaque numbers per well; in each case, the observed distribution showed a good match with the expected Poisson distribution (S2 Fig). Together with the observed linear relationship between plaque number and parasite dilution, these results convincingly confirm that—at least under limiting dilution conditions where plaque numbers can be accurately counted—each plaque usually or always derives from a single precursor parasite-infected erythrocyte and multiple plaques do not frequently arise from a single infected cell.

Use of the Plaque Assay for Phenotypic Analysis of a Replication-Defective *P. falciparum* Mutant

To explore the broader utility of the plaque assay, we applied it to the phenotypic characterization of a genetically modified *P. falciparum* clone known to display a partially-defined growth defect. *Plasmodium* merozoites are uniformly coated with a glycolipid anchored surface protein called MSP1. We have previously shown [8] that conditional genetic truncation of the *P. falciparum* MSP1 gene to remove its C-terminal membrane-anchoring domain results in merozoites that entirely lack surface-bound MSP1. These merozoites undergo highly inefficient egress from the host erythrocyte, with a resulting defect in parasite replication under static conditions *in vitro*. We predicted that this defect would manifest itself in the plaque assay as a reduction in the number and/or size of plaques. To test this prediction, a culture of highly synchronized ring-stage *P. falciparum* MSP1:loxPint parasites [8, 9], which possess a loxP-flanked 3' segment of their MSP1 gene and constitutively express a rapamycin (RAP)-inducible form of Cre recombinase (DiCre) [10], was divided into two. The cultures were then treated in parallel with either RAP (to induce DiCre-mediated gene excision) or DMSO (control solvent) only. The two treated cultures were washed, diluted identically to a theoretical ~75 parasitised cells per ml (15 parasites/well, 0.75% haematocrit), dispensed into flat-bottomed microplates and the

resulting plaques examined 14 days later. Imaged plaque numbers were determined by manual counting, whilst the pixel area of individual plaques was quantified using the Magic Wand tool of Adobe Photoshop CS5. As shown in Fig 2, plaques formed in the RAP-treated cultures were smaller and less numerous (mean = 199.3, SD = 9.2, n = 54) than those formed in the control wells (mean = 329.1, SD = 6.7, n = 205), with a two-tailed independent t-test revealing this difference to be highly significant ($t = 9.290$, d.f. = 257, $p < 0.0001$). The differences in mean plaque area values indicate a fitness defect associated with truncation of MSP1, consistent with the previously observed ~2-fold slower replication rate displayed by this mutant relative to the parental parasite clone in standard parasite growth assays [8]. Collectively, these results show that the plaque assay can be used as a simple means of rapidly characterizing even rather subtle effects of mutagenesis on parasite fitness.

Rapid Phenotypic Analysis of a Conditional Lethal *P. falciparum* Mutant

To further examine the applicability of the plaque assay, we used it to examine a second *P. falciparum* mutant in which the DiCre system was used to conditionally disrupt a gene predicted to be essential for *in vitro* growth. The malarial serine-rich antigen (SERA) family comprises a group of papain-like proteins encoded by genes with variable numbers of orthologues in all *Plasmodium* species examined [11]. Interest in the SERA proteins, which all share similarities with papain-family proteases, has arisen from evidence that they play roles in parasite egress and/or host cell invasion. Previous attempted direct gene disruption experiments using targeted homologous recombination have suggested that two of the nine *P. falciparum* SERA genes, SERA5 and SERA6, are essential in asexual blood stages [12, 13]. To extend our previous work indicating that SERA6 may have a function at egress [14], we produced transgenic *P. falciparum* parasites (called SERA6:loxP) in which the entire SERA6 locus was flanked by loxP sites (manuscript in preparation; J. Thomas, C. Collins and M. Blackman). These parasites were produced on the genetic background of the same DiCre-expressing parasite clone (called 1G5DC [10]) used to produce the MSP1:loxP parasites described above. A synchronous culture of a SERA6:loxP clone was divided into two and treated in parallel with either RAP to induce DiCre-mediated gene excision, or DMSO only. The treated cultures were washed, diluted identically to a theoretical ~75 parasites per ml (15 parasites/well), dispensed into flat-bottomed microplates and the resulting plaques imaged 14 days later. As shown in Fig 3A, the results demonstrated a dramatic difference between the control DMSO-treated cultures, which showed substantial numbers of plaques, and the RAP-treated parasites, in which only a single plaque appeared in one well. RAP-mediated gene excision in the *P. falciparum* DiCre system, whilst tightly regulated and highly efficient, is rarely complete in a treated population [8, 10]. This can result in heterogeneous parasite populations that contain both excised and non-excised parasites, making it difficult to unambiguously assess the effects of gene modification on parasite fitness in uncloned RAP-treated cultures. Based on the clear difference between the RAP-treated and control SERA6:loxP parasites in the plaque assay, we hypothesized that the single plaque formed in the RAP-treated cultures might be derived from a parasite that had not undergone excision of the floxed SERA6 gene. To test this, parasites from the plaque-positive well in the RAP-treated samples were expanded and analyzed by diagnostic PCR. This showed that these parasites indeed possessed an intact, non-excised genomic SERA6 locus (Fig 3B). These results convincingly demonstrate that loss of SERA6 expression produces non-viable parasites that are completely unable to produce plaques. Importantly, because the plaque assay allowed visualization of parasite fitness at the level of individual parasite clones, it enabled simple comparison of the RAP-treated and control cultures and allowed us to quickly demonstrate

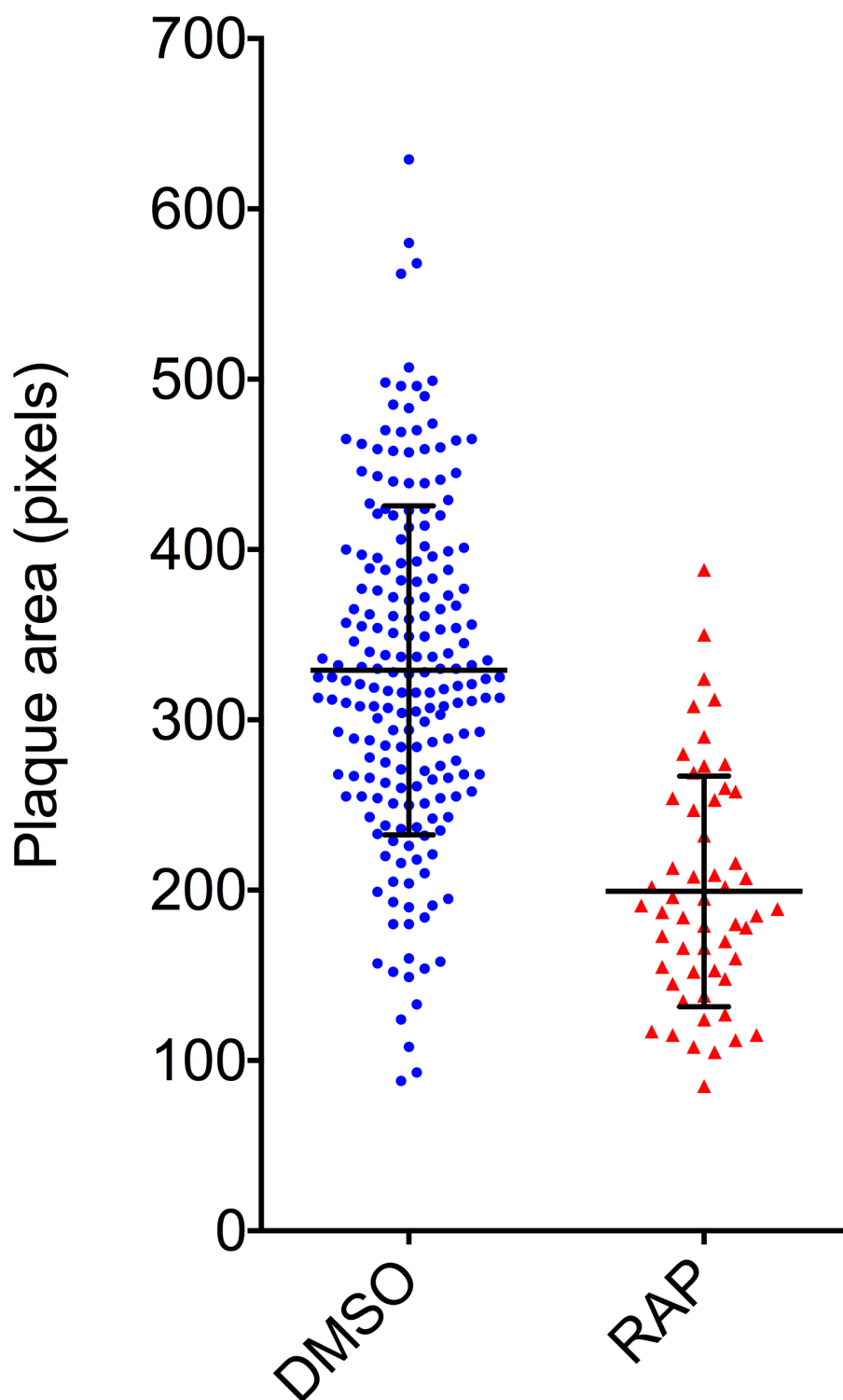


Fig 2. Phenotypic characterisation of an MSP1 mutant using the plaque assay. Scatter plots showing the distribution of plaque sizes obtained following treatment of MSP1:loxPint parasites with DMSO (control, mock-treated) or RAP to induce DiCre-mediated truncation of MSP1. Plaque numbers ($n = 205$ for the DMSO-treated samples and $n = 54$ for the RAP-treated samples) were counted manually. Plaque dimensions were quantified using the Magic Wand tool of Photoshop CS5 (Adobe). Horizontal bars indicate mean plaque area ± 1 SD.

doi:10.1371/journal.pone.0157873.g002

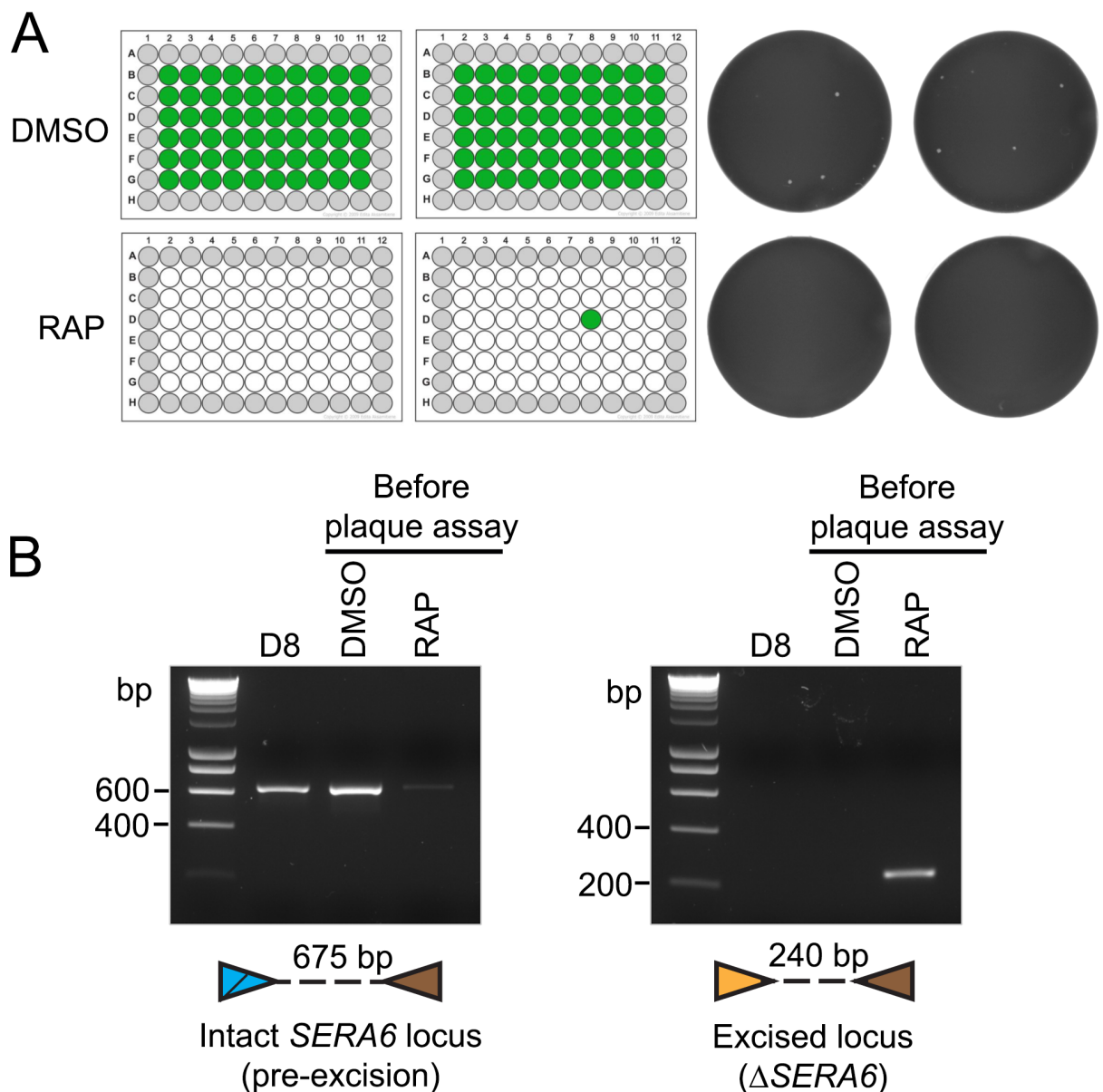


Fig 3. Rapid phenotypic characterization of a lethal conditional *P. falciparum* mutant using the plaque assay. (A) Left hand-side; schematic of the results of plaque analysis of RAP-treated and DMSO (mock)-treated *SERA6:loxP* parasites. Microplate wells coloured green indicate those that contained plaques 14 days following plating out the parasites at a theoretical 10 parasites/well. White wells contained no plaques (wells shown in grey were not used for the cloning). Whereas plaques were present in every well of the mock-treated culture, only a single plaque appeared in one well (well D8) of the RAP-treated culture. Right hand-side; example wells from the RAP-treated and control plates (green channel only of the scanned image shown to enhance plaque visibility, displayed as a grayscale image; see [Materials and Methods](#) for details). (B) Diagnostic PCR analysis of either the bulk *SERA6:loxP* parasite population immediately following RAP or DMSO-treatment (before plaque assay), or parasites expanded from well D8 of the +RAP plate. RAP-treatment significantly reduced the intact-*SERA6* locus-specific signal in the parasite population and resulted in appearance of a signal specific for the excised locus. Parasites rescued from well D8 of the RAP-treated parasites displayed a non-excised genomic architecture. The results strongly suggest that excision of the *SERA6* gene is lethal. Arrow-heads indicate the oligonucleotide primers used for PR analysis: blue, *SERA6*-34; yellow, JTS5synthF; brown, JTPbDT3'R (see [Materials and Methods](#) for primer sequences and PCR parameters). Expected sizes of the PCR amplicons are indicated.

doi:10.1371/journal.pone.0157873.g003

that the only parasites in the RAP-treated population capable of surviving were those rare parasites in which excision of the *SERA6* gene had not occurred.

Discussion

There has long been a need for a simple and robust plaque assay suitable for use with *P. falciparum*. Here we have developed such a system, using the simple expedient of culturing the parasite under static, limiting dilution conditions in human erythrocytes in flat-bottomed microplates. Visualisation of plaque formation was haematocrit-dependent, but we found that plaques were easily detectable within ~2 weeks under optimised conditions (0.75% haematocrit). Based on a mean human erythrocyte volume of $\sim 90 \times 10^{-15}$ L [15], we calculate that under the conditions used the settled erythrocytes formed layers that are at least 24 cells thick. This likely allows productive parasite proliferation and plaque growth in 3 dimensions, as opposed to the two dimensional plaque expansion allowed by the adherent erythrocyte monolayers used in previously-described *P. falciparum* plaque assays. The plaques, which presumably comprise predominantly haemoglobin-free erythrocyte ghosts, cell debris and haemozoin pigment, are completely stable under conditions of regular manual plate transferral to and from incubators, even without the use of semi-solid media, probably because the small volumes in each microplate well limit liquid movement and consequent disturbance of the erythrocyte layer. Use of larger wells or flasks would probably destabilise plaques, which may explain why the formation of these structures has not previously been described. Plaque integrity and stability was further ensured in the assay by not replacing the medium during the 2–3 week period of the assay. Although this may have the effect of limiting parasite growth rates, we found that up to a plaque density of at least ~340 plaques per well, plaque frequency correlated with starting parasite density, implying that—at least within this parasitaemia range—plaque formation is not adversely affected by plaque density. Plaques produced by clonal parasite populations were not completely homogeneous in size or shape, but this is a feature of many plaques, including those produced by *Toxoplasma*. The *P. falciparum* plaques are easily visible using an inverted light microscope and, similar to viral plaques [16], can be rapidly imaged with minimal disturbance using an inexpensive, commercially available document scanner, avoiding the need for laborious photographic imaging of individual wells. Indeed, using a high resolution (4,800 dpi) flat-bed scanner in transmission light mode, we found that we were able to scan and document up to 3 plates simultaneously in a single TIFF image, allowing for rapid documentation of multiple plates. The ready availability, low cost and lack of user training required to use a flat-bed scanner makes this a highly accessible, medium-throughput means of documenting the results of assays.

The *P. falciparum* plaque assay has important advantages compared to those commonly used for viruses and other intracellular pathogens such as *Toxoplasma*. Mature erythrocytes do not replicate, so there is no need to take into account changes in host cell confluency over the course of the assay. Furthermore, there is no requirement to fix and stain the cells before plaque analysis. The ability to easily visualise parasite clonal growth has other advantages. In our laboratory we now regularly use the approach for rapid limiting dilution cloning of *P. falciparum* lines without the need for laborious determination of starting parasitaemia values, simply by performing serial dilutions of a parasite culture across a plate then picking clones only from those wells that contain a single plaque. It is important to transfer these clones from the plaque assay plates into cultures containing fresh erythrocytes before the plaque assay cultures begin to undergo generalised erythrocyte lysis, which we found usually occurs between 16–21 days after initiating the plaque assay. The plaque assay may be applicable to the study of other *Plasmodium* species that can be cultivated *in vitro*, though our attempts to optimise it for use with

culture-adapted lines of *P. knowlesi* [17] were not encouraging; in this case the plaques formed were irregular and indistinct, perhaps a result of the well-documented greater extracellular motility and longevity of free *P. knowlesi* merozoites [18, 19] which may allow more rapid dispersal of released merozoites within the wells than occurs with *P. falciparum* (unpublished data, J. Thomas).

Reverse genetic technologies have become increasingly important in malarial research over the past 2 decades. With the relatively recent introduction of Cas9-mediated mutagenesis [20, 21] and conditional gene modification technologies in *P. falciparum* [10, 22–24] we believe that the development of simple assays such as that described here is particularly timely. The plaque assay has multiple applications; here we have shown that it can be used to rapidly phenotype genetic mutants without the need for complex equipment or facilities (e.g. flow cytometry or spectrophotometry), but it also will be invaluable for the calculation of kill-curves with anti-parasite drugs and antibodies, for the selection of drug-resistant or fast-growing mutants, for facile comparison of parasite fitness under a range of growth conditions (e.g. in different defined media) and numerous other uses. Differences in parasite growth rate are presumably propagated over the course of the 4–7 cycles required to produce visible plaques, providing some advantages over traditional growth assays in that the cultures do not need to be passaged or fed. Importantly, the assay complements other widely used approaches for quantitation of parasite growth, including light microscopy, flow cytometry and assays based on expression of parasite proteins or enzymes [25, 26], incorporation of fluorescent dyes [27, 28] or radioactive hypoxanthine [29]. Although not explored here, the assay may be suitable for scaling up to high throughput image-based screens using high content microscopy. We believe the plaque assay will prove a valuable addition to the experimental tools available for studying this important pathogen.

Materials and Methods

P. falciparum Maintenance and Manipulation

Parasites (wild type clone 3D7, the DiCre-expressing clone 1G5DC [10], and MSP1:loxPint or SERA6:loxP parasites which were produced on the 1G5DC genetic background [8, 9]) were routinely cultured at 37°C in human erythrocytes at 1–4% haematocrit in RPMI 1640 (Life Technologies) supplemented with 2.3 gL⁻¹ sodium bicarbonate, 4 gL⁻¹ dextrose, 5.957 gL⁻¹ HEPES, 0.05 gL⁻¹ hypoxanthine, 0.5% (w/v) Albumax II, 0.025 gL⁻¹ gentamycin sulphate, and 0.292 gL⁻¹ L-glutamine (complete medium) in a low oxygen atmosphere as previously described [30, 31]. Parasitaemia was never allowed to exceed 10%. Human blood was obtained from anonymised donors through the UK National Blood Transfusion service and was used within 2 weeks of receipt. No ethical approval is required for its use. Routine microscopic examination of parasite growth was performed by fixing air-dried thin blood films with 100% methanol before staining with 10% Giemsa stain (VWR international) in 6.7 mM phosphate buffer, pH 7.1. For routine microscopic determination of parasitaemia values, at least 5,000 erythrocytes were examined. Haematocrit values were determined using a haemocytometer (Marienfield; 0.1 mm depth and 0.0025 mm² area), assuming that 100% haematocrit equates to 1 x 10¹⁰ normocytes mL⁻¹. For synchronisation, mature schizont stage parasites were isolated on cushions of 70% (v/v) Percoll (GE Healthcare) adjusted to isotonicity as described [32, 33]. Lysis of mature forms of the parasite to enrich for ring forms after invasion was performed by suspending parasites in 5% (w/v) D-sorbitol [32, 34].

Generation of Transgenic *P. falciparum* Clones and PCR Analysis

Production of the *P. falciparum* MSP1:loxPint clone which possess a *loxP*-flanked 3' segment of the *MSP1* gene and constitutively expresses a RAP-inducible form of Cre recombinase has

been described [8, 9]. Production of the SERA6:loxP line was achieved by introducing a *loxP* site into the genome directly downstream of the *SERA6* locus in the 1G5DC clone, which already possesses a genomic *loxP* site downstream of the *SERA5* locus. Details of the approach used to generate the SERA6:loxP parasites will be provided separately (manuscript in preparation; J. Thomas, C. Collins and M. Blackman). When desired, transgenic parasites were treated with RAP (100 nM final concentration) or DMSO (control vehicle, 1% v/v) for 4 h, as described [10]. Genomic DNA was prepared from treated parasites for diagnostic PCR analysis as described [10]. The intact modified *SERA6* locus was amplified by PCR using oligonucleotide primers JTPbDT3'R (5'-TTACAGTTATAAATACAATCAATTGG-3') plus SERA6-34 (5'-GTCTGGAAGAAGAACGTTGCCGCCGCGAGACACAACACTGACTTCATG-3'). The excised locus (following DiCre-mediated recombination) was amplified using primers JTPbDT3'R plus JTS5synthF (5'-GAATGCTATTTCTGCTACGTG-3'). For diagnostic PCR analysis a Kappa2G Fast HotStart ReadyMix PCR Kit (Kappa Biosystems) was used. Thermal cycle conditions for amplification of the intact modified *SERA6* locus were: initial denaturation at 95°C for 60 sec, followed by 20 cycles of 95°C for 10 sec, 51°C for 10 sec and 72°C for 15 sec, with a final extension at 72°C for 60 sec. Thermal cycle conditions for amplification of the excised locus were: initial denaturation at 95°C for 60 sec, followed by 25 cycles of 95°C for 10 sec, 55°C for 10 sec and 72°C for 15 sec, with a final extension at 72°C for 60 sec.

Imaging, Documentation and Quantitation of Plaque Formation

To allow plaque formation, synchronous parasite cultures at ring stage were diluted to the desired densities in complete medium with human erythrocytes at haematocrits between 0.5% and 2%, then dispensed into flat-bottomed 96 well microplates (Costar 3596, Corning NY, USA; 0.32 cm² growth area per well, diameter 6.38 mm) using 200 µl culture per well. Plates were incubated in gassed, humidified sealed modular incubator chambers (Billups-Rothenberg, CA, USA). To limit evaporation, only the inner 60 wells of microplates were used for cultures whilst sterile phosphate buffered saline was added to the outer wells of plates. Plaque formation was assessed routinely by microscopic examination using a Nikon TMS inverted microscope (40x magnification). When desired, plaque formation was documented without opening the plates using a Perfection V750 Pro scanner (Epson) in top-down transmission light mode, saving images as 4,800 dpi RGB TIFF files. Usually 3 microplates were imaged simultaneously with the scanner. Plaque visibility was generally enhanced by using the 'Split channels' function of the Fiji distribution of ImageJ [35] to split the RGB TIFF file into red, green and blue data channels, then using the green channel image for quantification of plaque dimensions, since this provided maximum contrast between plaques and the surrounding erythrocyte layer. Plaques were counted by visual examination of the images and plaque size quantified using the Magic Wand tool in Adobe Photoshop CS5 using a tolerance setting value of 32. This tool delineates the perimeter of selected plaques and calculates plaque area in pixels. No area value was recorded for apparent plaques that could not be delineated by the tool. Statistical analysis (linear regression analysis by analysis of covariance and t-test) was performed using GraphPad Prism 7 software (CA, USA).

Supporting Information

S1 Fig. The experimentally observed frequency distribution of plaques in the plaque assay follows a Poisson distribution.

(PDF)

S2 Fig. Plaque frequency shows a linear correlation with parasite number. (PDF)

Acknowledgments

We are indebted to Paco Pino (University of Geneva) for initial observations and discussions leading to this study.

Author Contributions

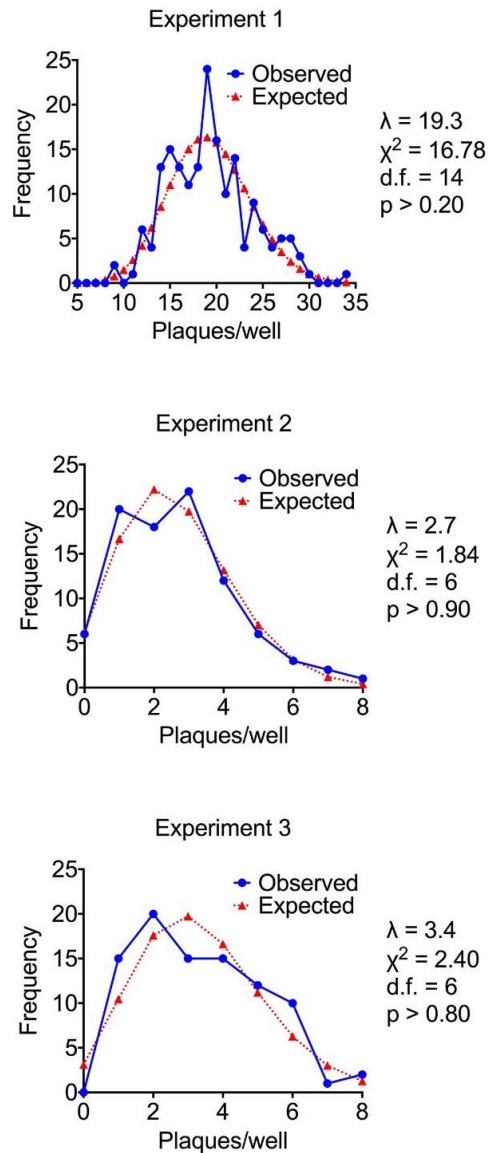
Conceived and designed the experiments: JAT CRC SD DB MJB. Performed the experiments: JAT CRC SD FH. Analyzed the data: JAT CRC SD FH MJB. Contributed reagents/materials/analysis tools: AG DB ED. Wrote the paper: JAT MJB.

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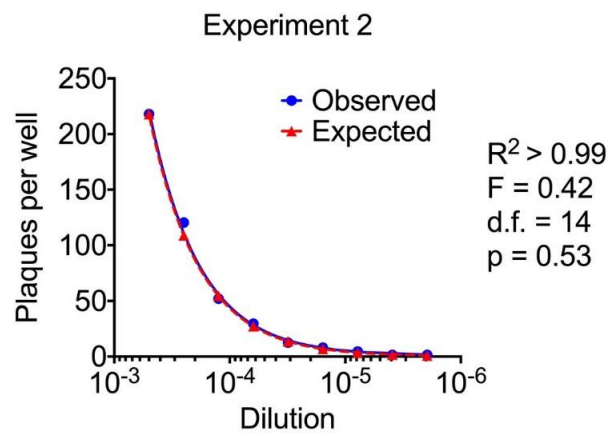
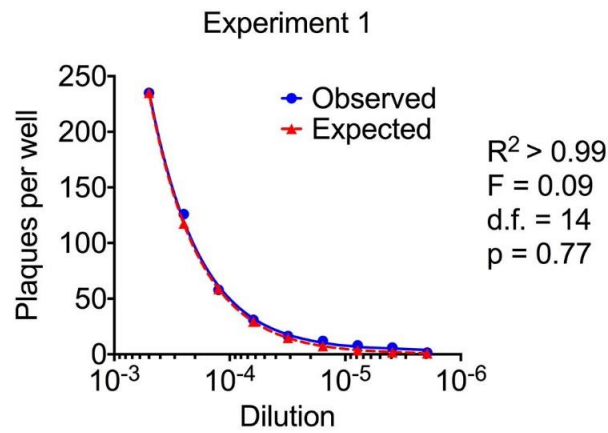
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S1 Fig. The experimentally observed frequency distribution of plaques in the plaque assay follows a Poisson distribution. Depicted are plaque assay data from three independent experiments carried out on different days in which serially diluted 3D7 *P. falciparum* cultures (0.75% haematocrit) were cultured in flat-bottomed 96-well microplates as described in Materials and Methods. Plaques were enumerated at 14 days and mean values (λ) calculated. Experimentally observed plaque frequency values are shown plotted (blue) alongside the expected frequency values of a Poisson distribution with the same mean value (red). Chi-squared goodness-of-fit tests showed no significant deviation of observed distributions from the expected distributions. Chi-squared (χ^2) values, number of degrees of freedom (d.f) and p-values are indicated in each case. Note that for the purpose of statistical analysis, those categories where expected values were less than 5 were pooled to create a single category.



S2 Fig. Plaque frequency shows a linear correlation with parasite number. Depicted are plaque assay data from two independent experiments carried out on different days in which 2-fold serial dilutions of 3D7 *P. falciparum* cultures (0.75% haematocrit) were cultured in flat-bottomed 96-well microplates as described in Materials and Methods. Plaques were enumerated at 14 days. Plots of observed mean plaque frequency against dilution (blue) are shown alongside plots of plaque frequencies expected if there is a linear inverse correlation between plaque number and dilution (red). To establish the fit between the plots, linear regression was performed by analysis of covariance (ANCOVA) in GraphPad Prism. The results indicate a strong linear inverse correlation between dilution and plaque density in the assay, with no significant difference between the observed and expected curves. Values of the statistical data (R^2 , F statistics, number of degrees of freedom and p values) are shown.

